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ANNALES MEDICINAE EXPERIMENTALIS ET BIOLOGIAE FENNIAE

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VOL. 28 1950 FASC. 1

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Annales Medicinae Experimentalis et Biologiae Fenniae

is a direct continuation of the *Acta Societatis Medicorum Fennicae ›Duodecim‹*, 1919—1930 (Vols. I—XII) and the *Acta Societatis Medicorum Fennicae ›Duodecim‹, Ser. A*, 1931—1946 (Vols. XIII—XXIV).

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Annales Medicinae Experimentalis et Biologiae Fenniae

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FROM THE DEPARTMENT OF ANATOMY, UNIVERSITY OF HELSINKI

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CYTOCHEMICALLY DEMONSTRABLE GLYCOGEN IN HUMAN WHITE BLOOD CELLS

By

OLAVI ERÄNKÖ

(Received for publication September 21, 1949)

Iodine reaction and Best's carmine stain are the classical methods for cytochemical demonstration of glycogen. They have been of great significance in histological research but more specific and sensitive methods have to a great extent replaced them. The basis of these newer methods is Bauer's reaction (1), in which the tissue polysaccharides are visualized with the Schiff leucofuchsin solution after treatment with cold chromic acid. In the recent modifications either periodic acid has been used instead of chromic acid (2, 3, 4, 5) or the Schiff reagent has been changed to some other aldehyde reagent, such as ammoniacal silver nitrate (e.g. 6, 7), alkaline silver-methenamine (8), or silver diamino hydroxide (9). The periodic acid-leucofuchsin (PAL) method has been said to be the method of choice and able to demonstrate heavier glycogen infiltrations than the original Bauer stain; it gives, further, a positive reaction in some Bauer negative structures (5). None of the cited methods is quite specific but, in the general opinion, positive reaction which is abolished with previous saliva digestion indicates the presence of polysaccharides.

Several authors (10, 11, 12, 13, 14), with various cytochemical methods for glycogen, have demonstrated the presence of polysaccharides in the cytoplasm of the neutrophilic leucocyte, either diffusely or in granular form. Reports concerning other blood cells are contradictory.

The eosinophilic granules have been said to stain with Best's carmine or iodine (10, 11). The eosinophilic leucocytes of man (13),

of rhesus monkey (12), and of rat (12), are, however, not stainable with the Bauer method. Very recently, Wachstein (14), whose paper was not available to me until this study was completed*), observed a faintly positive PAL reaction in the cytoplasm of human eosinophilic leucocytes, although only polymorphonuclear leucocytes and blood platelets showed consistent staining. The eosinophilic granules did not stain.

The blood platelets and lymphocytes of man and of monkey are entirely Bauer negative (12, 13). With iodine, with Best's carmine (11), or whith the PAL method (14), a brilliantly stained center and a paler outer border is to be seen in the platelets, and some small granules in some lymphocytes.

This study deals with the presence and minute localization of Bauer and PAL positive material in human white blood cells.

MATERIAL AND METHODS

The material consisted of smears of peripheral blood from healthy human individuals. The blood was obtained by finger puncture. Smears were dried for 2 to 10 min. and some of them were fixed in 99 per cent alcohol for 2 to 10 min. A part of fixed smears were coated with celloidin.

For demonstrating the presence of polysaccharides both the Bauer method (1) and the PAL method (4) were used. The reagents were prepared from chemicals of analytical grade and they were used only once. The basic fuchsin was manufactured by the National Aniline Division, N.Y., U.S.A. Before performing the reaction, control smears were incubated in undiluted saliva or in water at room temperature (appr. 20°C) for 1 hr.

Stained specimens were mounted first in water and then in balsam. They were examined in both media. For high-power examination Zeiss's apochromate 120X, N.A. 1.30, and Cooke Troughton and Simss's phase-contrast achromate 95X, N.A. 1.30, were serviceable. Identification of various cell types was possible, before and after staining, with the aid of the phase-contrast equipment. Noting the coordinates in the vernier scale of the mechanical stage, it was possible to examine the appearence of some particular cell throughout the staining process. Counterstaining was not necessary.

*) I am grateful to Dr. Wachstein for a reprint, received 5th March, 1950.

OBSERVATIONS

Neutrophilic Leucocytes. — In agreement with earlier investigations the cytoplasm of great majority of neutrophilic leucocytes was found to be Bauer positive. The nucleus always remained unstained. With the PAL technic the cytoplasm was even more deeply stained than with the Bauer method. No striking differences in the intensity of staining were observed between unfixed, fixed, and fixed, celloidinized smears.

Under the 120X apochromate objective and in transmitted light the cytoplasm of well-flattened cells appeared purplewhite spotted but the exact location of the stain was impossible to state. The phase-contrast microscope was more useful. The purple recolored leucofuchsin shined brilliantly green (possibly due to the uncorrected condenser of the lamp) and the image was very sharp. Owing to the minuteness of the granulation, it was difficult to state whether the granules or the intergranular spaces were stained. It seemed rather likely, however, that the specific granules of the neutrophilic leucocyte were presented by the minute white »holes» in the colored cytoplasm. This view was supported by the fact that apparent pseudopodia in the periphery of some cells were thoroughly colored.

Neutrophilic leucocytes in the saliva pretreated control smears showed purple color after neither the Bauer method nor the PAL technic. After similar pretreatment in water, the cells were definitely stained in celloidinized smears. In the uncoated smears, nevertheless, incubation in water before the staining abolished considerable amounts of material giving positive reactions for carbohydrates. The loss of stainability after water treatment was somewhat greater in the alcohol-fixed than in the unfixed specimens.

Eosinophilic Leucocytes. — In Bauer stained cells no or occasional very weak color could be seen in the cytoplasm. In smears treated with the PAL technic, the cytoplasm was always consistently colored, the nucleus remaining unstained. The intensity of the color was weaker than that in the neutrophilic leucocytes.

In transmitted light it was already possible to see that the stain was located in the narrow spaces between the unstained refringent granules, Fig. 1. The image was, however, disturbed

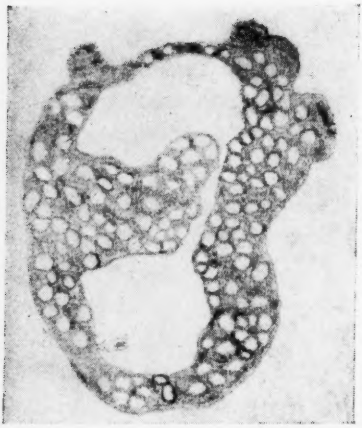

Fig. 1. — Camera lucida drawing of a human eosinophilic leucocyte stained with the periodic acid-leucofuchsin method. Transmitted light. The intergranular spaces are colored, the granules unstained.

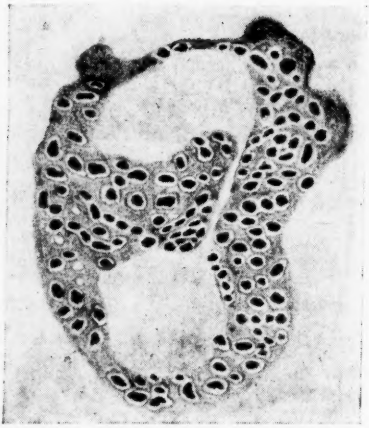

Fig. 2. — The same cell as seen in the phase-contrast microscope. The granules look dark owing to their high refringence; the intergranular substance is colored. × Ca. 3000.

by the refraction phenomena due to the strong refringence of the granules (15). The smears mounted in balsam were less troublesome, in this respect.

In the phase-contrast microscope a sharp figure was to be seen. The granules were dark red centrally and they were surrounded by a colorless circle, Fig. 2. The PAL positive intergranular substance appeared bright green. The red granules and the surrounding white halos together were of the size of the specific granules as seen, *e.g.*, in Sudan black stained smears. In some cells relatively long and thin red rods were seen between the two nucleal parts.

After saliva treatment the eosinophilic leucocytes stained still in the celloidinized smears with the PAL method, although somewhat more weakly.

Lymphocytes and *Monocytes*. — The periphery of these mononuclear cells showed often a weak diffuse color with the PAL method. In addition, stained granules could often be observed in the cytoplasm. They were usually few, but in some large monocytes numerous fine granules were to be seen. In the control smears, the mononuclear cells were colorless.

Blood Platelets. — Like the mononuclear cells, the blood plate-

lets were Bauer negative. The PAL method stained the platelets weakly purple. Centrally there was one or two large strongly PAL positive granules and, more peripherally, some minute granules. The large granules stained still after saliva treatment.

CONCLUSIONS

The material in the neutrophilic leucocytes stainable with the Bauer method and with the PAL technic was abolished with saliva but not with water, in celloidinized smears. Presuming that this specific action of saliva is due to its content of polysaccharide-splitting enzymes the stainability evidently indicates the presence of polysaccharides, probably glycogen, in the neutrophilic leucocytes.

In uncoated smears, water was able to dissolve histochemically demonstrable glycogen. Therefore, coating of the smears is apparently necessary. According to Lillie (5), however, phosphate buffer solution the p_H of which is about 7 does not remove glycogen. — It is not clear why the stainable material was more easily dissolved by water in fixed than in unfixed smears. Wachstein (14) observed that short saliva treatment influenced less the unfixed than the fixed neutrophilic leucocytes and he attributed this to the denaturating action of alcohol on the cytoplasmic proteins.

In the neutrophilic leucocytes, the glycogen seemed to be located rather in the intergranular spaces than in the granules. This is in agreement with the view of Rheingold and Wislocki (13). In their preparations the glycogen was shifted by fixation to one side of the cell, in contrary to the granules, which, in the investigators' opinion, seemed to not contain glycogen, therefore. A direct evidence has been given by the present author (16): in smears stained for both lipids and glycogen the granules are black and the intergranular spaces purple.

The intergranularly located PAL positive substance in the eosinophilic leucocytes disappeared not with the saliva treatment. The polysaccharidous nature of it seems thus doubtful. It is to be mentioned, however, that the densely packed granules, between which the stainable material lies, contain lipids insoluble in alcohol. They may, perhaps, protect glycogen against the dissolving effect of saliva.

The granules in the blood platelets were stainable with the

PAL method after saliva digestion also. Material responsible for the stainability is, therefore, not certainly glycogen, which possibly caused the diffuse weak color of the platelets and the stainability of lymphocytes and of monocytes.

SUMMARY

Bauer's chromic acid-leucofuchsin method and the recent periodic acid-leucofuchsin technic were used to study the distribution of cytochemically demonstrable glycogen in human white blood cells.

The cytoplasm of the neutrophilic leucocytes contains material, probably glycogen, stainable with both methods and specifically digestable with saliva. The material is apparently intergranularly located. Other types of cells are Bauer negative.

Intergranular spaces in the eosinophilic leucocytes stain with the periodic acid method; the stain is not entirely abolished with previous saliva treatment. Central granules in the blood platelets behave similarly.

Weak color in the cytoplasm of lymphocytes and of monocytes as well as diffusely in the blood platelets, in smears stained with the periodic acid method, is possibly caused by their glycogen content.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

ANTIBIOTICS IN LICHENS II

By

K. O. VARTIA

(Received for publication October 17, 1949)

Reports on titre values pertaining to the antibiotic property of crystalline lichen acids are relatively scarce. Barry (8) 1946 found for diploicin a growth-inhibiting titre at 1: 100,000 affecting diphteria and tubercle bacilli. The lichen acid isolated by Marshak (14) from *Ramalina reticulata* inhibited the growth of the human tubercle bacillus in a dilution of 1: 20,000—1: 50,000; later on he found that this substance was identical with the d-usnic acid isolated from *Cladonia sylvatica* (15). Stoll, Renz, and Brack (19), on the other hand, reported much higher inhibitory values for l-usnic acid obtained by them as a derivate of *Cetraria islandica:* human TB 1: 320,000—1: 800,000, *Mycob. phlei* 1: 125,000, *Streptococcus pyogenes* and *Staphylococcus aureus* 1: 100,000; equally Bustinza and Lopez (9) for d-usnic acid from *Usnea barbata:* human TB 1: 500,000, *Mycob. phlei* 1: 100,000, *Staphylococcus aureus* 1: 20,000 —1: 50,000. Cavallito et al. (10) published in 1948 a series of tests performed with lactones of aliphatic acids, among them l-protolichesteric acid, mainly contained in *Cetraria islandica*, and its derivates l-lichesteric and l-dihydroprotolichesteric acids. The results obtained were consistent as to their extent with those yielded by other aliphatic lactones. The bacteriostatic minimal concentrations in these three substances were as follows in millimols per cc $\times 10^4$: (in parentheses the calculated titres equalised to 10,000): TB 0.03—0.06 (1: 1,000,000—1: 600,000), *Streptoc. pyogenes* 0.6—1.0

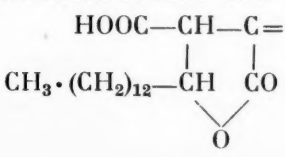
(1: 50,000—1: 30,000), *Staphylococcus aureus* 0.3 (1: 100,000), *Cl. Welchii* 0.015—0.15 (1: 2,000,000—1: 200,000), S. typhi > 30 (> 1: 1,000). Shibata et al. (17) obtained 1948 inhibitory titres 1: 160,000—1: 320,000 for several optic isomeres of usnic acid against *Staphylococcus aureus* and avian tubercle bacillus. In 1949 Marshak et al. (15) tested d-usnic acid and its 31 derivates, as well as vulpic acid, on different TB strains; their growth was prevented by d-usnic acid, racemic usnic acid and monoacetyl usnic acid at 1: 100,000. In my earlier work (20) I obtained with l-usnic acid extracted from *Cladonia alpestris* a growth-inhibiting effect on two TB strains at a titre of 1: 60,000. Klosa (13) reported for evernic acid and usnic acid a growth-inhibiting titre of 1: 1,000,000 affecting Streptococci, Staphylococci, and human tubercle bacilli.

PRESENT INVESTIGATION

The object of this study is to examine the growth-inhibiting effect of nine lichen acids[1] on eleven bacteria. An one per cent sodium salt solution was prepared from each acid by titrating with 0.1-N NaOH solution. Basic dilution 1: 5,000 (1.0 one per cent solution, 49.0 broth; the pH was adjusted at 7.5). The bacteria to be cultivated were diluted from broth with a 24 hours growth in the following way: Streptococcus, Sarcina and Pneumococcus 1: 500, Staphylococcus, Megatherium, Diphteria and Subtilis 1: 5,000, Coli, Paratyphus, Proteus and Pyocyaneus 1: 50,000. The inoculation quantity was 0.05 cc of the bacterial dilution. The results were read after 18 hours. (+) = responses varied at different times.

[1] The lichens were collected in the vicinity of Helsinki (*Cetraria islandica, Cladonia uncialis, Haematomma ventosum*), and at Laukaa (*Evernia prunastri, Parmelia physodes, Parmelia saxatilis*). I am very grateful to Professor E. Häyrén, who has made the determination of the species, and to miss Toive Pietarinen for carrying out most of the chemical work.

D-PROTOLICHESTERIC ACID $C_{19}H_{32}O_4$



Preparation from *Cetraria islandica* essentially according to (21). The lichen was extracted with ether (Soxhlet), after evaporation purified with benzene, dissolved in cold ether, the fumarprotocetraric acid remaining undissolved. Crystallised from benzene and glacial acetic acid: colourless leaves, mp. 105—6° C. (Asahina: 105—6° C).

$[\alpha]_D^{20} = +11.9°$ (10.9 mg in 1 cc chloroform). (Asahina: + 11.4—12.8° at several temperatures).

Titration: 54.8 mg crystals (acetone, phenolphtalein); 1.70 ml of 0.1-N NaOH were consumed. Molecular weight as monobasic acid 322 (Calculated M = 324).

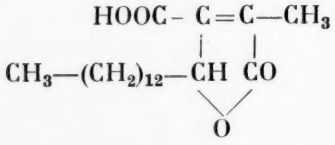
1 per cent aqueous solution, containing equivalent amount of NaOH, of which 1: 50 dilution was made to the broth (Basic solution 1: 5,000, pH 7.5).

TABLE 1

	1: 5,000	1: 10,000	1: 20,000	1: 40,000	1: 80,000	1: 160,000	1: 320,000	1: 640,000	1: 1,280,000	Control	Control
Sarcina lutea Nyb.	−	−	−	−	−	−	(+)	+	+	+	+
Staph. aureus 1055	−	−	−	−	−	(+)	+	+	+	+	+
Streptoc. β-hemol. Lep.	−	−	−	−	−	−	−	+	+	+	+
Pneumoc. typ. 2. 2421	−	−	−	−	−	−	+	+	+	+	+
Coryneb. dipht. mitis 2065/414491	−	−	−	−	−	−	(+)	(+)	+	+	+
B. megatherium 13/7	−	−	−	−	−	−	(+)	+	+	+	+
B. subtilis 12 R.	−	−	−	−	(+)	+	+	+	+	+	+
E. coli 647	+	+	+	+	+	+	+	+	+	+	+
S. parat. B. 21905/46	+[1]	+	+	+	+	+	+	+	+	+	+
Proteus vulgaris 1045	+[1]	+	+	+	+	+	+	+	+	+	+
Ps. aeruginosa 20/9	+[1]	+	+	+	+	+	+	+	+	+	+

[1] growth weaker as in controls

D-LICHESTERIC ACID $C_{19}H_{32}O_4$



Preparation according to (6): d-protolichesteric acid (mp. 103—4° C) was heated with acetanhydride in an oil-bath at 100—105° C for 2 hours. The precipitate occurring on the addition of water was filtered and crystallised from glacial acetic acid: colourless leaves, mp. 123—4° C. (Asano: 123.5° C).

Titration: 53.5 mg crystals (ethanol, phenolphtalein); 1.60 ml of 0.1 —N NaOH were consumed. Molecular weight as monobasic acid 334 (Calculated M = 324).

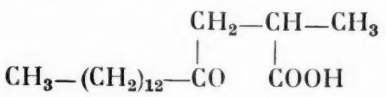
1 per cent aqueous solution, containing equivalent amount of NaOH, of which 1: 50 dilution to the broth (Basic solution 1: 5,000, pH 7.5).

TABLE 2

	1: 5,000	1: 10,000	1: 20,000	1: 40,000	1: 80,000	1: 160,000	1: 320,000	1: 640,000	1: 1,280,000	Control	Control
Sarcina lutea Nyb.	−	−	−	−	−	−	−	+	+	+	+
Staph. aureus 1055	−	−	−	−	−	+	+	+	+	+	+
Streptoc. β-hemol. Lep.	−	−	−	−	−	−	−	+	+	+	+
Pneumoc. typ. 2. 2421.....	−	−	−	−	−	−	+	+	+	+	+
Coryneb. dipht. mitis 2065/414491 ..	−	−	−	−	−	−	−	+	+	+	+
B. megatherium 13/7	−	−	−	−	−	−	+	+	+	+	+
B. subtilis 12 R.....	−	−	−	−	−	(+)	+	+	+	+	+
E. coli 647	+	+	+	+	+	+	+	+	+	+	+
S. parat. B. 21905/46	+[1]	+	+	+	+	+	+	+	+	+	+
Proteus vulgaris 1045	+[1]	+	+	+	+	+	+	+	+	+	+
Ps. aeruginosa 20/9	+[1]	+	+	+	+	+	+	+	+	+	+

[1] growth weaker as in controls

LICHESTERYLIC ACID $C_{18}H_{34}O_3$



Preparation according to (7): d-lichesteric acid (mp. 123—4° C) was boiled for 2 hours in a water bath with 0.1—N KOH. It was precipitated with hydrochloric acid, the precipitate was washed in water and crystallised from methanol: white needles, mp. 79—81° C. (Asano: 83—84° C).

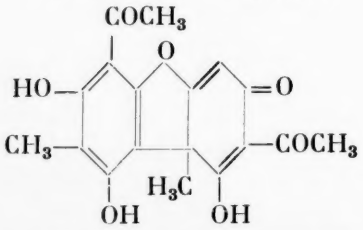
Titration: 46.8 mg crystals (ethanol, phenolphtalein). 1.44 ml of 0.1-N NaOH were comsumed. As monocarbonic acid M = 298 (Calculated M = 325).

1 per cent solution, containing equivalent amount of NaOH, from which basic solution 1: 5,000, pH 7.5.

TABLE 3

	1: 5,000	1: 10,000	1: 20,000	1: 40,000	1: 80,000	1: 160,000	1: 320,000	1: 640,000	1: 1,280,000	Control	Control
Sarcina lutea Nyb.	−	−	−	−	(+)	+	+	+	+	+	+
Staph. aureus 1055	−	−	−	+	+	+	+	+	+	+	+
Streptoc. β-hemol. Lep.	−	−	−	−	+	+	+	+	+	+	+
Pneumoc. typ. 2. 2421.....	−	−	−	−	+	+	+	+	+	+	+
Coryneb. dipht. mitis 2065/414491 ..	−	−	−	−	(+)	(+)	+	+	+	+	+
B. megatherium 13/7	−	−	−	−	−	+	+	+	+	+	+
B. subtilis 12 R.....	−	−	−	−	−	+	+	+	+	+	+
E. coli 647	+	+	+	+	+	+	+	+	+	+	+
S. parat. B. 21905/46	+	+	+	+	+	+	+	+	+	+	+
Proteus vulgaris 1045	+	+	+	+	+	+	+	+	+	+	+
Ps. aeruginosa 20/9	+	+	+	+	+	+	+	+	+	+	+

L-USNIC ACID $C_{18}H_{16}O_7$



Preparation from *Cladonia uncialis* essentially according to (21): the lichen was extracted with acetone (Soxhlet), after evaporation treated with ether, thamnolic acid remaining undissolved. Crystallised from benzene; acicular crystals, mp. 197—8° C. (Asahina: 202° C, Zopf: 196° C).

$[\alpha]_D^{20} = -492°$ (11.9 mg in 1.0 cc of chloroform). (Asahina (several results): + 490—500° for d-usnic acid, Siintola et al.: −452°).

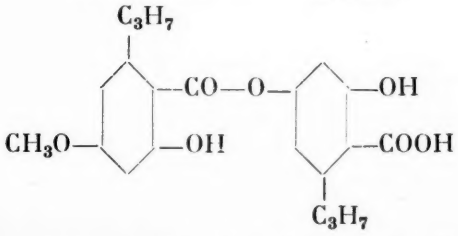
Titration: 69.6 mg crystals (ethanol, phenolphtalein). 2.00 ml of 0.1-N NaOH were consumed. As monocarbonic acid M = 348 (Calculated M = 344).

1 per cent aqueous-alcohol solution (40 per cent ethanol), containing equivalent amount of NaOH, i.e. ethanol contents of basic solution (1: 5,000) made to the broth were 0.8 per cent. The first 2 tubes of the dilution series were somewhat turbid owing to the poor solubility of the acid.

TABLE 4

	1: 5,000	1: 10,000	1: 20,000	1: 40,000	1: 80,000	1: 160,000	1: 320,000	1: 640,000	1: 1,128,000	Control	Control
Sarcina lutea Nyb.	−	−	−	−	−	−	(+)	+	+	+	+
Staph. aureus 1055	−	−	−	−	(+)	+	+	+	+	+	+
Streptoc. β-hemol. Lep.	−	−	−	−	−	+	+	+	+	+	+
Pneumoc. typ. 2. 2421.....	−	−	−	−	(+)	+	+	+	+	+	+
Coryneb. dipht. mitis 2065/414491 ..	−	−	−	−	−	(+)	+	+	+	+	+
B. megatherium 13/7	−	−	−	−	−	−	+	+	+	+	+
B. subtilis 12 R.....	−	−	−	−	−	(+)	+	+	+	+	+
E. coli 647	+	+	+	+	+	+	+	+	+	+	+
S. parat. B. 21905/46	+	+	+	+	+	+	+	+	+	+	+
Proteus vulgaris 1045	+	+	+	+	+	+	+	+	+	+	+
Ps. aeruginosa 20/9	+	+	+	+	+	+	+	+	+	+	+

DIVARICATIC ACID $C_{21}H_{24}O_7$



Preparation according to (21, 3): extracted with cold ether from *Haematomma ventosum*, crystallised from 60 per cent ethanol, acetic acid, benzene and acetone, last crystallisation from ethanol after an absorption with animal charcoal: thin white needles, mp. 136° C. (Asahina: 137° C).

Titration: 53.7 mg crystals (ethanol, phenolphtalein). 1.60 ml of 0.1-N NaOH were consumed. As monocarbonic acid M = 336. (Calculated M = 388).

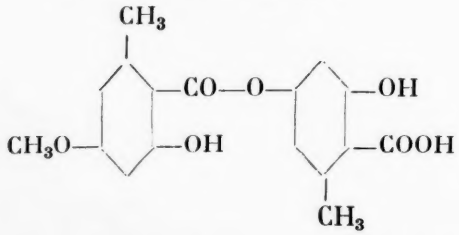
1 per cent aqueous solution is made by adding an equivalent amount of NaOH and 10 per cent ethanol; from it 1: 50 dilution (Basic solution 1: 5,000, pH 7.5).

1 per cent solution, even when stored in a refrigerator, gradually assumes a reddish tint, and it seems that its activity disappears simultaneously.

TABLE 5

	1: 5,000	1: 10,000	1: 20,000	1: 40,000	1: 80,000	1: 160,000	1: 320,000	1: 640,000	1: 1,280,000	Control	Control
Sarcina lutea Nyb.	−	−	−	−	−	+	+	+	+	+	+
Staph. aureus 1055	−	−	−	+	+	+	+	+	+	+	+
Streptoc. β-hemol. Lep.	−	−	−	−	−	−	+	+	+	+	+
Pneumoc. typ. 2. 2421.....	−	−	−	−	+	+	+	+	+	+	+
Coryneb. dipht. mitis 2065/414491 ..	−	−	−	−	−	+	+	+	+	+	+
B. megatherium 13/7	−	−	−	−	−	−	+	+	+	+	+
B. subtilis 12 R.....	−	−	−	−	−	+	+	+	+	+	+
E. coli 647	+	+	+	+	+	+	+	+	+	+	+
S. parat. B. 21905/46	+	+	+	+	+	+	+	+	+	+	+
Proteus vulgaris 1045	+	+	+	+	+	+	+	+	+	+	+
Ps. aeruginosa 20/9	+	+	+	+	+	+	+	+	+	+	+

EVERNIC ACID $C_{17}H_{16}O_7$



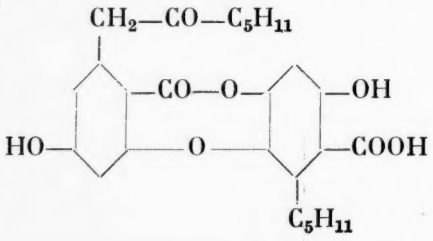
Preparation from *Evernia prunastri* essentially according to (12). The lichen was extracted with ether (Soxhlet); after evaporation treated with chloroform and dissolved after filtration in hot ethanol, the atranorin remaining undissolved, while the evernic acid is precipitated after the cooling of ethanol. Crystallised from acetone: small white crystals, mp. 167° C (Hesse: 169° C).

Titration: 76.7 mg crystals (ethanol, phenolphtalein). 2.25 ml of 0.1-N NaOH were consumed. As monocarbonic acid M = 341 (Calculated M = 332)

1 per cent aqueous-alcohol solution (20 per cent ethanol) was prepared, with an equivalent amount of NaOH, of which a 1: 50 dilution was made to the broth (basic solution 1: 5,000, pH 7.5). The 1 per cent solution gradually assumed a reddish tint.

It was revealed in the series of tests performed that evernic acid could not arrest the growth of any bacteria used in the tests at a dilution of 1: 5,000, but appeared to weaken the growth of Sarcina, Streptococcus, Megatherium, Diphtheria and Subtilis at 1: 5,000—1: 10,000.

PHYSODIC ACID $C_{26}H_{30}O_8$

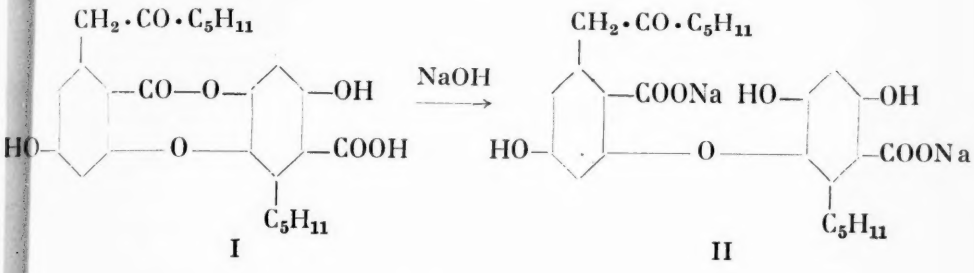


Preparation from *Parmelia physodes* essentially according to (4). The lichen was extracted with ether (Soxhlet). Atranorin precipitated after the evaporation of ether was filtered off and the ether

distilled dry. The remainder was subjected to chloroform and crystallised from methanol: white crystals, mp. 195° C (Asahina: 205° C, Zopf: 200—202° C, Hesse: 190—192° C).

Titration: 100.0 mg crystals (acetone, phenolphtalein). 4.22 ml of 0.1-N NaOH were consumed. As dicarbonic acid M = 471 (Calculated M = 470).

The bibasity of physodic acid is due to the opening of the lactonic ring when titrating already at room temperature the physodic acid (I) chancing to the isophysodic acid (II) (4):

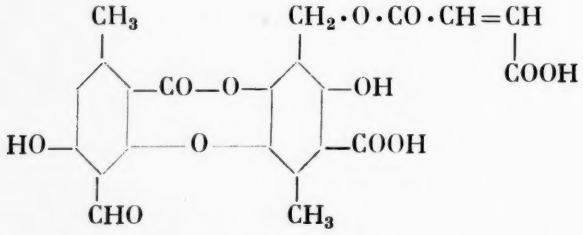


I II

TABLE 6

	1: 5,000	1: 10,000	1: 20,000	1: 40,000	1: 80,000	1: 160,000	1: 320,000	1: 640,000	1: 1,280,000	Control	Control
Sarcina lutea Nyb.	−	−	−	−	+	+	+	+	+	+	+
Staph. aureus 1055	−	−	−	+	+	+	+	+	+	+	+
Streptoc. β-hemol. Lep.	−	−	−	−	(+)	+	+	+	+	+	+
Pneumoc. typ. 2. 2421.....	−	−	−	−	+	+	+	+	+	+	+
Coryneb. dipht. mitis 2065/414491 ..	−	−	−	−	−	−	+	+	+	+	+
B. megatherium 13/7	−	−	−	−	+	+	+	+	+	+	+
B. subtilis 12 R.....	−	−	−	−	+	+	+	+	+	+	+
E. coli 647	+	+	+	+	+	+	+	+	+	+	+
S. parat. B. 21905/46	+	+	+	+	+	+	+	+	+	+	+
Proteus vulgaris 1045	+	+	+	+	+	+	+	+	+	+	+
Ps. aeruginosa 20/9	+	+	+	+	+	+	+	+	+	+	+

FUMARPROTOCETRARIC ACID $C_{22}H_{16}O_{12}$



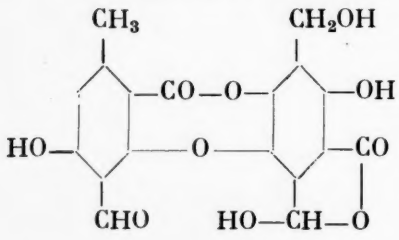
Preparation from *Cetraria islandica* essentially according to (21, 5). After extraction with ether (page 8) the lichen was extracted with acetone. Crystallises from acetone: greyish-white crystals which do not dissolve but contract and turn red, carbonise 260—270° C.

Titration: 88.9 mg crystals (acetone, bromthymolblue); 3.80 ml of 0.1-N NaOH were consumed. Molecular weight as dibasic acid 468 (Calculated M = 472).

1 per cent aqueous solution was made, containing 32 per cent ethanol and equivalent amount of NaOH, of which 1: 50 dilution to the broth (Basic solution 1: 5,000, pH 7.5). 1 per cent solution is pale yellow.

The acid is powerless to arrest the growth of any bacteria used in the tests at a dilution of 1: 5,000, but seems to retard the growth of pyocyaneus: the broth dilution of 1: 5,000 of 18 hours growth is unmistakable turbid, but the green tint is lacking.

SALAZINIC ACID $C_{18}H_{12}O_{10}$



Preparation from *Parmelia saxatilis* essentially according to (21, 2). The lichen was extracted with ether when atranorin was dissolved. The lichen thus prepared was extracted with acetone; crystallised from acetone: white crystals of a slightly reddish tint,

forming a paper-like mass. The melting point could not be determined, instead the crystals slowly changed to a reddish brown and carbonised at about 260—270° C.

Titration did not yield reliable values.

1 per cent solution resembling that of fumarprotocetraric acid, brownish-red.

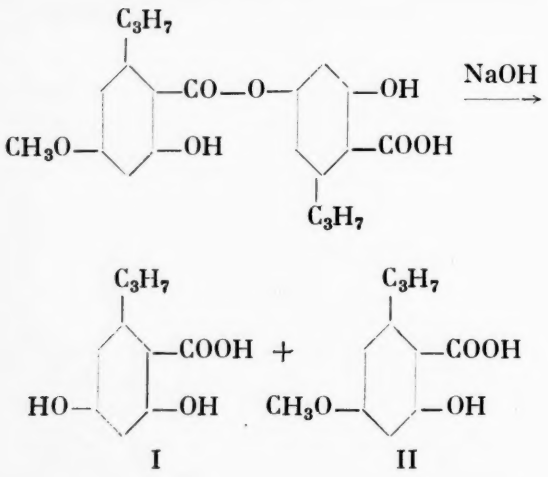
The basic solution 1: 5,000 was unable to inhibit the growth of the bacteria experimented with, but the green tint of Pyocyaneus in this dilution was clearly slighter than in the controls.

DISCUSSION

The nine lichen acids under examination represent, according to Asahina (1), four different types: d-protolichesteric, d-lichesteric and lichesterylic acids fatty acid-lactone group, usnic acid cumarone derivates, divaricatic and evernic acids depsides and physodic, fumarprotocetraric and salazinic acid depsidones. The chemical differences being so great, the results are surprisingly similar. No exact comparisons can be made on the basis of tests performed in this way, since even different test series performed with the same substance yield variable results. It must be said furthermore that the broth used is by no means an adequate nutrient medium, particularly for streptococci and pneumococci.

Among representatives of the *fatty acidlactone group* the d-protolichesteric and d-lichesteric acids proved to be — contrary to the opinion of Stoll et al. (19) — at least equally effective as the usnic acid. It is noteworthy that lichesterylic acid which, in contradistinction to those already mentioned, has no lactone ring but only one keto- and one methylgroup in a saturated aliphatic chain, is clearly active, although weaker than the roregoing. The existence of a lactone (or some other) ring is evidently not of decisive significance in determining the antibiotic nature of a substance, as already surmised by Cavallito et al. (10).

Depsides. — The weakened activity of divaricatic acid preserved as a sodium salt solution seems to be due to the disintegration of this acid into divaric (I) and divaricatinic (II) acid (11):



Is it still unsettled whether the weak antibiotic effect of evernic acid is due to a similar disintegration, or whether it is inactive per se.

Depsidones. — The physodic acid in a sodium salt solution is no depsidone anymore, the lactone ring having opened (page 15). The fumarprotocetraric and salazinic acid seem to have some slightly retarding effect on the growth of Pyocyaneus, but no effect on Gram-positive bacteria.

SUMMARY

Nine crystalline lichen acids were tested on eleven species of bacteria. Of them six acids (d-protolichesteric, d-lichesteric, lichesterylic, l-usnic, divaricatic and physodic acids) were found to possess unmistakable antibiotic properties against Gram-positive bacteria. The d-protolichesteric and d-lichesteric acids inhibit slightly the growth of *S. paratyphi B*, *Proteus vulgaris* and *Pseudomonas pyocyaneus aeruginosa*, too. The fumarprotocetraric and salazinic acids seem to have some slightly retarding effect on the growth of *Ps. pyocyaneus aeruginosa*, but no effect on Gram-positive bacteria.

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Aided by a grant from Emil Aaltonen Foundation.

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SWARMING OF PROTEUS VULGARIS AND ITS INHIBITION IN ROUTINE DIAGNOSTICS WHEN USING BROM CRESOL PURPLE PLATE

By

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(Received for publication May 31, 1949)

It is a well-known fact that *Proteus vulgaris* and *Proteus Morgani* grow as a thin, hardly distinguishable layer over the surface of solid culture media. They differ from each other, among other things, in the temperature they grow in. The latter grows in 20—29° C and not in 37° as the former. Thus no swarming of *Proteus Morgani* can be ascertained in routine diagnosties. On the other hand *Proteus vulgaris* is not at all uncommon in normal faeces and urine specimens, nor in the presence of intestinal infections.

If *Proteus vulgaris* is inoculated into the centre of an ordinary 2½ per cent agar plate, there will be, according to Russ-Münzer (3), slight growth visible in four hours. During the following two hours the first swarming ring grows round the colony. 10—30 μ long Proteus rods (normally 1—3 μ long) which are often rich in grains, work outwards from the centre towards the periphery. They form branching protuberances which join neighbouring offshoots with their points, while the empty spaces between them are filled from the centre of the colony. In this way a zone that is 1—1.5 cm wide is formed in six hours round the colony which has grown at the place of inoculation. After that time the overgrowth stops for 2 hours, during which time the thin, hardly distinguishable layer grows thicker, and then again a 2 hours' swarming period starts. The zones of overgrowth which in this way have been formed during periods of 4 hours' gradually cover the whole surface of the culture medium.

According to Lominski & Lendrum (2) »the swarming of Proteus is a motility stimulated and orientated by the negative chemotactic action of its metabolites. Swarming occurs only under conditions affording a spatial gradient of these metabolites. The active substance can be obtained cell-free».

MATERIAL

During the period of 1947—1948 5,263 faeces and urine specimens were examined at the Institute of Serology and Bacteriology in the University of Turku.

Technique. — On the same day as the specimens arrived they were cultured direct on brom cresol purple plates (primary plates) and into Kauffmann's enriching culture medium (1). The diametre of the plates was 10 cm, and the compound of the brom cresol purple culture medium was as follows:

Meat water	1000 g
Peptone	5 g
Agar	20 g
Sodium chloride	3 g
Lactose Bacto	15 g
Brom cresol purple 1.6% alcohol solution	1 cc

Pains were taken to test the primary brom cresol purple plates, if possible, after a 24 hours' period of incubation. Some of Kauffmann's enriching medium was also cultured after a growth for 24 hours on brom cresol purple plates (secondary plates), which were tested the following day. For lack of raw materials and scarcity of available plates two specimens were always cultivated on the same plate at our institute.

In the following the term brom cresol purple plate will be abbreviated brcp-plate.

In the above-mentioned time *Proteus vulgaris* grew over the surface of the solid culture medium in 586 cases, covering all the other growths, both pathogenic and non-pathogenic. Since, for the above-mentioned reasons, two specimens were cultivated on the same plate, this figure is only a relative one. During the same time 668 positive paratyphoid and 4 positive typhoid specimens were diagnosed. The occurrence of positive typhoid-paratyphoid cultures and swarmings of Proteus in the different months are shown in Table No 1 and Fig. 1.

TABLE 1

OCCURRENCE OF POSITIVE TYPHOID-PARATYPHOID CULTURES AND SWARMINGS OF PROTEUS VULGARIS IN VARIOUS MONTHS DURING THE YEARS 1947—1948

Month	Number of Specimens	Positive Typhoid-para-typhoid Cultures	%	Swarming of Proteus on Primary Plate	Swarming of Proteus on Sec. Plate	%
January ..	357	73	20.1		33	9.3
February ..	276	50	18.1		30	10.9
March	416	88	21.2		20	4.8
April	475	67	14.1	2	46	9.7
May	360	38	10.6		54	15.0
June	356	15	4.2		32	8.7
July	333	21	6.3	1	19	5.7
August ..	415	1+22	5.5		40	9.6
September	576	69	11.9		71	12.3
October ..	602	103	17.3		83	12.2
November	519	3+50	10.2		89	15.2
December	578	72	12.4	3	69	11.9
Total	5263	4+668	12.8	6 = 0.1 %	586	11.1

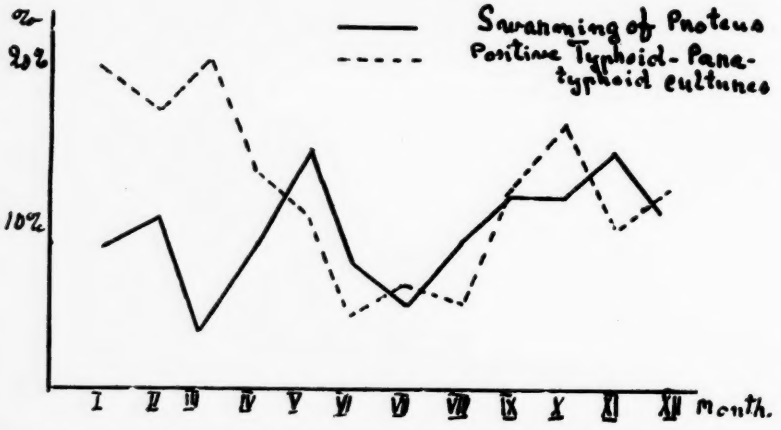

Fig. 1. — Frequency of the swarming of Proteus and the positive typhoid-paratyphoid cultures per month.

The curve showing the swarming of Proteus and that showing the positive cultures are parallel to a great extent. Thus it seems that the frequency of *Proteus vulgaris* culminates at about the

same time as, or perhaps just a little later than, the positive typhoid-paratyphoid cultures.

The material shows further that the major part of the swarmings of *Proteus vulgaris* appeared on cultures taken from a Kff-enriching medium, in other words on the secondary plates. Only in 6 cultures, or 0.1 per cent of the cases, swarming was ascertained on the primary plate. Thus it is quite obvious that Kauffmann's enriching culture medium is a compound that enriches *Proteus vulgaris*.

INHIBITION OF THE SWARMING OF PROTEUS VULGARIS

The above-mentioned characteristic of *Proteus vulgaris* to swarm over the surface of a solid culture medium, which in our series took place in 11.1 per cent of the cases, is a great hindrance in studying the bacterial flora of urinary and faecal cultures. A number of substances have been recommended against overgrowth, such as morphine, chloral hydrate, luminal sodium, alcohol, sodium azide (NaN_3), phenol, 6 per cent agar, and bile acide salts (4, 5, 6).

Using a brcp-plate I titrated, chloral hydrate, sodium azide, Na-taurocholate, and Na-desoxicholate in order to ascertain their inhibiting effect on the swarming of *Proteus vulgaris*, at the same time noting their effect on some of the most common pathological Gram negative bacilli in the intestinal flora. The results can be seen from Fig. 2.

Chloral hydrate and sodium azide proved to be useless because they had to be used in such a high concentration that the growth of pathogenic bacteria was entirely inhibited or curtailed to such an extent that it caused great difficulties. In connection with the tests and with specimens of faeces that had been prepared artificially (a Proteus strain and typhoid, paratyphoid, or Flexner strains were added to the faeces) control tests were carried out which showed that with 0.1 per cent Na-desoxicholate and 0.5 per cent Na-taurocholate concentration a sufficient inhibition of the swarming of *Proteus vulgaris* could be obtained. Further it showed that the above-mentioned bile acide salts specifically check the swarming of *Proteus vulgaris* without affecting the growth of the other strains of bacteria used in the tests, and also without affecting the O- and H-agglutinations of the typhoid, paratyphoid, and Flexner strains.

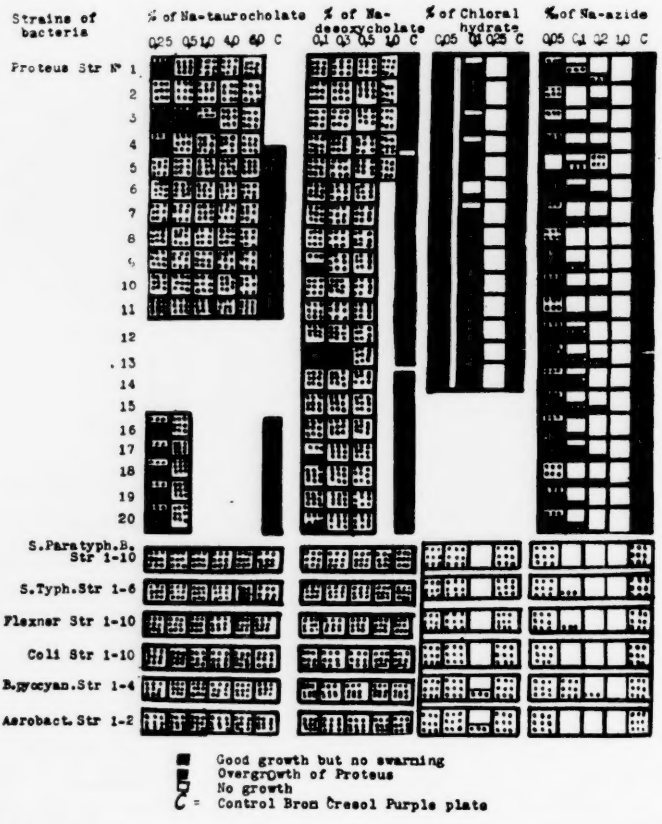

Fig. 2. — Inhibition of swarming by Na-taurocholate, Na-desoxicholate, chloral hydrate, and Na-azide on brom cresol purple plate, and influence of these chemicals on the growth of some of the commonest Gram negative intestinal bacilli.

Since much less Na-desoxicholate is needed than Na-taurocholate, the use of the former is also economical.

Since our series proved that Kauffmann's enriching culture medium also is extremely efficient in the case of *Proteus vulgaris*, we tried to cause an inhibition of the swarming of *Proteus vulgaris* on the secondary plate by adding either tauro- or desoxicholate to the culture medium and by substituting the bile in it by these chemicals. By using artificially prepared specimens of faeces I, realised however, that 0.1 per cent desoxicholate and 0.5 per cent taurocholate in Kauffmann's enriching culture medium were not sufficient to cause any inhibiting influence on cultures taken

from Kauffmann's culture medium onto a secondary plate. But on the other hand it was found that, when the brcp-plate contained tauro- or desoxicholate in the above-mentioned quantities, an inhibition was caused in the swarming of Proteus, and that the greatest inhibiting effect was obtained when there were no bile acids in Kauffmann's culture medium.

COMPARISON BETWEEN BROM CRESOL PURPLE DESOXICHOLATE PLATE AND BRCP-PLATE

On the basis of my preliminary tests, 1,291 urinary and faecal specimens were examined by using simultaneously brcp-plates and plates to which 0.1 per cent Na-desoxicholate had been added. The last-mentioned plate will be called here the brcpd-plate.

As the swarming of Proteus was ascertained only in 0.1 per cent of the primary plates of my series, parallel plates were only used in the case of Kauffmann's enriching culture medium.

The result of the research on 1,291 urinary and faecal specimens will be seen from Table 2.

TABLE 2

COMPARISON BETWEEN CULTURES TAKEN FROM KAUFFMANN'S ENRICHING MEDIUM ONTO PARALLEL PLATES

Number of cultures with Proteus swarmings on brcp-plates			146	(11.31%)
Number of cultures with Proteus swarmings on brcpd-plates			1	(0.08%)
Number of cultures positive only on brcp-plates, but negative on brcpd-plates ..	4	(3.2%)		
Number of cultures positive only on brcpd-plates, but negative on brcp-plates	11	(8.7%)		
Number of cultures with Proteus swarmings on brcp-plates but with positive cultures on brcpd-plates	15	(11.9%)		
Number of positive cultures on both brcp- and brcpd-plates	96	(73.2%)		
Total number of positive typhoid-paratyphoid cultures	126		126	(9.76%)
Number of negative cultures			1018	(78.85)%
Number of specimens examined			1291	

Table 2 shows that the swarming of Proteus is almost completely inhibited on brcd-plates, and that this inhibition increases the number of positive cultures by 11.9 per cent. Moreover, it is worth noting that there were 8.7 positive cultures per cent on brcpd-plates only, while the parallel plates remained negative. Thus

additional positive cultures can be obtained in altogether 20.6 per cent of the cases by using brcpd-plates.

It is therefore obvious that it is worth while to add 0.1 per cent Na-desoxicholate to the brcp-plate and to use it when searching for typhoid-paratyphoid bacilli in urine and faeces. When the doubtful cases due to the swarming of Proteus are excluded and when the hospitals thus arrive at a more accurate diagnosis, the period of treatment will be shorter in many cases where Proteus is found. This is an economical advantage for both the hospital and the patient.

SUMMARY

1. It seems that the frequency of *Proteus vulgaris* culminates almost simultaneously with, or perhaps slightly later than, the positive typhoid-paratyphoid cultures.

2. When examining the inhibiting influence of chloral hydrate, sodium azide, sodium taurocholate, and sodium desoxicholate on the swarming of Proteus on brom cresol purple plates the writer ascertained that only the salts of the above-mentioned bile acids were serviceable and that by using 0.1 per cent Na-desoxicholate and 0.5 per cent Na-taurocholate a sufficient inhibition of the swarming of Proteus was obtained.

3. In the said concentration paratyphoid, typhoid, and Flexner bacilli grow well on the brom cresol purple desoxicholate plate, and there is no visible inhibition of their H- and O-agglutinations.

4. As the swarming of Proteus is inhibited on brcpd-plates the number of positive cultures is increased by 11.9 per cent. Taking into account the positive cultures appearing on brcpd-plates only, while the parallel plates remained negative, additional positive cultures are obtained in altogether 20.6 per cent of the cases.

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FROM THE WOMEN'S CLINIC AND THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

STUDIES ON DAILY CHANGES IN THE PROTEIN VALUES OF FEMALE SERUM

By

ANNA-LIISA ALHA

(Received for publication September 8, 1949)

I wanted to find out to what extent the protein values of the female serum vary in the blood samples taken at different times of the day.

It has been maintained that the total protein values of the serum are somewhat lower in the evening than in the morning. According to Bergmann (3) this decrease after an ordinary working day is 4 $^0/_{00}$.

The investigation was carried out in the manner described in my earlier article (1).

My series consisted of 20 healthy nurses, of whom 3 were pregnant (Cases 2, 6, and 15).

The blood samples were taken at 8 o'clock in the morning, after breakfast at 12, before dinner at 4 (at 16) p.m., and after dinner at 7 (at 19) p.m. Table 1 shows the results.

It will be noticed that there are considerable daily changes in the protein values, but the direction of the daily changes cannot be determined from these results with certainty. Fig. 1 gives the mean values of each determination made at the same time of the day.

Probability calculations have been carried out to compare the results of the investigation.

TABLE 1

Test No.	Hr.	Non-prot. N. of Ser.	Tot. prot. %	Alb. %	Glob. %	A/G	Test No.	Hr.	Non-prot. N. of Ser.	Tot. prot. %	Alb. %	Glob. %	A/G
1.	8	30	7.355	4.363	2.992	1.458	2.	8	26	6.505	3,234	3.171	1.019
	12		7.653	4.065	3.588	1.131		12		6.505	3,740	2.765	1.352
	16		7.513	4.030	3.483	1.157		16		6.163	3.688	2.475	1.490
	19		7.425	4.100	3.325	1.233		19		6.418	3.985	2.433	1.638
3.	8	30	6.935	4.188	2.747	1.525	4.	8	30	6.568	3.260	3.308	1.016
	12		7.723	4.225	3.498	1.208		12		7.530	3.808	3.722	1.023
	16		7.460	4.485	2.975	1.507		16		8.073	4.100	3.973	1.032
	19		7.285	4.345	2.940	1.481		19		7.583	3.480	4.103	0.848
5.	8	30	7.600	4.328	3.272	1.322	6.	8	30	6.568	3.225	3.343	0.965
	12		8.335	4.380	3.955	1.107		12		6.620	3.400	3.220	1.055
	16		7.810	4.275	3.535	1.206		16		6.708	3.670	3.038	1.208
	19		7.670	4.223	3.447	1.219		19		6.673	3.575	3.098	1.154
7.	8	30	7.443	3.838	3.605	1.064	8.	8	34	7.785	4.674	3.111	1.506
	12		7.670	4.065	3.605	1.127		12		7.155	3.970	3.185	1.246
	16		7.425	4.258	3.167	1.344		16		7.558	4.373	3.185	1.373
	19		7.550	4.520	3.030	1.490		19		6.928	4.110	2.818	1.458
9.	8	30	7.093	4.066	3.027	1.344	10.	8	30	8.060	4.503	3.557	1.266
	12		7.250	4.398	2.852	1.542		12		7.653	4.768	2.885	1.653
	16		7.110	4.515	2.595	1.739		16		7.898	4.503	3.395	1.326
	19		7.005	4.380	2.625	1.668		19		7.233	4.188	3.045	1.375
11.	8	30	7.688	3.742	3.946	0.949	12.	8	34	7.698	4.393	3.305	1.335
	12		7.145	3.575	3.570	1.001		12		7.033	4.466	2.627	1.671
	16		7.373	3.488	3.885	0.898		16		7.663	4.198	3.465	1.212
	19		7.355	3.768	3.587	1.050		19		8.083	4.145	3.938	1.053
13.	8	30	7.263	4.519	2.744	1.647	14.	8	34	7.206	4.329	2.977	1.454
	12		7.669	4.013	3.656	1.097		12		7.063	4.406	2.657	1.658
	16		7.688	4.238	3.450	1.222		16		7.813	4.494	3.319	1.354
	19		7.375	4.275	3.100	1.379		19		7.063	4.338	2.725	1.592
15.	8	34	6.688	3.813	2.875	1.326	16.	8	30	6.625	3.888	2.737	1.421
	12		6.500	3.425	3.075	1.096		12		7.500	4.538	2.962	1.532
	16		6.875	4.144	2.731	1.517		16		6.750	4.013	2.637	1.522
	19		7.000	4.231	2.769	1.528		19		6.875	4.113	2.762	1.489
17.	8	34	7.000	3.393	3.607	0.941	18.	8	34	7.225	3.969	3.256	1.219
	12		7.250	4.075	3.175	1.873		12		6.963	3.794	3.169	1.197
	16		6.975	4.006	2.969	1.349		16		6.906	4.194	2.712	1.546
	19		6.813	4.300	2.513	1.711		19		7.244	4.231	3.013	1.404
19.	8	34	7.488	3.863	3.625	1.066	20.	8	30	8.000	3.750	4.250	0.882
	12		7.363	3.775	3.588	1.052		12		7.612	3.650	3.962	0.921
	16		7.032	4.006	3.026	1.324		16		7.563	3.419	4.144	0.825
	19		7.500	3.950	3.550	1.113		19		7.625	3.575	4.150	0.861

The average daily change, which is the mean value of the variation range sums, can be seen from Table 2. The variation range is the difference between the greatest and the smallest value.

In an earlier investigation (2) I have dealt with the errors due to the technique. Table 3.

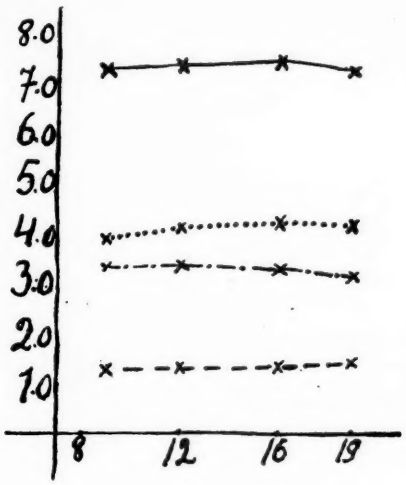

Fig 1.

——— Tot. Prot. %
..... Alb %
—·—·— Glob %
— — — Alb/Glob.

If we assume that all values fall inside the limits which are the mean value $\pm 3 \times$ the standard error of the mean value, we find that the average changes caused by technical errors in this investigation are, reckoning with greatest standard errors of the mean value:

Tot. Prot.	Alb.	Glob.
0.180	0.176	0.240

When comparing the average changes caused by technical errors with the average daily changes which I obtained in my investigation, we notice that the latter are so much bigger that it is very probable that there occur daily changes in the serum protein values. In order to make clear the nature of these changes

TABLE 2

Test Subject	Tot. Prot.	Alb.	Glob.	A/G.
1.	0.298	0.333	0.597	0.327
2.	0.342	0.751	0.738	0.619
3.	0.788	0.297	0.751	0.317
4.	1.505	0.840	0.715	0.184
5.	0.735	0.157	0.683	0.215
6.	0.140	0.445	0.305	0.243
7.	0.245	0.682	0.575	0.426
8.	0.857	0.704	0.367	0.260
9.	0.245	0.449	0.433	0.395
10.	0.827	0.580	0.672	0.387
11.	0.543	0.280	0.377	0.152
12.	1.050	0.261	1.311	0.618
13.	0.425	0.506	0.912	0.550
14.	0.750	0.165	0.662	0.304
15.	0.500	0.806	0.344	0.432
16.	0.875	0.650	0.325	0.111
17.	0.437	0.907	1.094	0.932
18.	0.338	0.437	0.544	0.349
19.	0.468	0.231	0.599	0.272
20.	0.437	0.331	0.288	0.096
	11.805	9.812	12.373	7.187
The average daily change	0.590	0.490	0.619	0.359

TABLE 3

STANDARD ERROR OF THE MEAN VALUE IN THE DETERMINATION OF THE SERUM PROTEIN FRACTIONS ACCORDING TO ALHA. 3 SUBJECTS, 10 DETERMINATIONS OF EACH

Test Subject	Tot. Prot. %		Alb. %		Glob. %		Alb./Glob.
	Mean Value	Standard Error	Mean Value	Standard Error	Mean Value	Standard Error	Mean Value
1.	7.238	0.030	4.618	0.023	2.620	0.038	1.764
2.	8.030	0.023	4.637	0.024	3.393	0.034	1.370
3.	5.747	0.026	3.325	0.029	2.422	0.040	1.370

I have grouped the maximum and minimum divisions in Tables 4 and 5.

The only statement we can make on the basis of these tables is that the maximum value of the globulin fraction, as well as the minimum of the albumin fraction, is possible to obtain in the morning.

TABLE 4

DIVISION OF MAXIMUM VALUES

Time by Clock	8	12	16	19
Tot. prot. ..	4.5	7.5	5	3
Alb.	4	4	6	6
Glob.	9.5	7	1.5	2
Alb./Glob. ..	5	6	5	4

TABLE 5

DIVISION OF MINIMUM VALUES

Time by Clock	8	12	16	19
Tot. Prot. ..	7	3.5	6	3.5
Alb.	9	5	3	3
Glob.	5	5	6	4
Alb./Glob. ..	7	8	3	2

In Fig. 2, I have described the correlation between the daily changes of the albumin and globulin fractions. It shows that the albumin and globulin fractions very probably are dependent on each other.

The material of this investigation is too limited to allow any conclusions concerning the nature of the daily changes, or to say whether they are incidental, or whether there are certain similarities there.

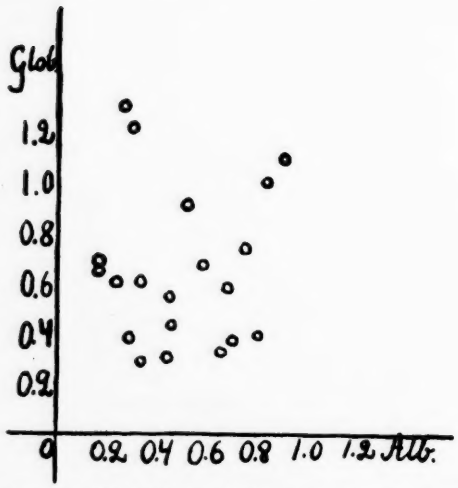

Fig. 2. Correlation of the daily variations of the alb. and glob. fractions. 20 subjects. o one test.

SUMMARY

The investigation on the daily changes of the serum protein values in 20 women showed that the daily changes were comparatively small. Probability calculations showed that the albumin values reached their minimum and the globulin values their maximum in the morning and that there probably is a correlation between the albumin and globulin fractions.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

ON THE PROPORTION OF ALBUMIN AND GLOBULIN FRACTIONS OF THE SERUM OF NORMAL MEN DETERMINED BY THE METHANOL PRECIPITATION METHOD

By

ANNA-LIISA ALHA

(Received for publication September 20, 1949)

In my investigation (1) concerning the serum protein fractions of normal women determined by the methanol precipitation method of Pillemer and Hutchinson (3), I obtained the following results:

	Tot. Prot.%	Alb.%	Glob.%	A/G
Limit values	6.568—8.387	3.260—4.818	2.534—4.385	0.760—1.807
Mean values	7.332	4.190	3.318	1.285
Standard deviations	0.459	0.395		

In this paper I shall give an account of a similar investigation which I have carried out with 40 normal men.

The subjects were medical students and members of laboratory staff in Helsinki, aged about 20—35 years.

Table 1 shows the results of my investigation.

The figures corresponding to those concerning women were as follows:

	Tot. Prot.%	Alb.%	Glob.%	A/G
Limit values	6.600—8.138	3.750—5.213	2.225—3.480	1.065—2.342
Mean values	7.531	4.579	2.949	1.572
Standard deviations	0.369	0.344		

Somewhat differing opinions occur in the literature about the proportions of the protein fractions in women and men. Though the determinations have been made by the neutral salt

TABLE 1

N:o	Tot. Prot. %	Alb.%	Glob. %	Alb/Glob.	Non-prot. N. of Ser.
1.	7.056	4.219	2.837	1.487	30
2.	7.313	3.869	3.444	1.065	30
3.	7.000	3.925	3.075	1.276	30
4.	7.500	4.538	2.962	1.530	30
5.	7.188	4.031	3.157	1.276	30
6.	7.313	4.600	2.713	1.704	30
7.	7.625	4.744	2.881	1.646	30
8.	7.813	4.850	2.963	1.637	30
9.	7.438	4.781	2.657	1.799	30
10.	7.125	4.188	2.837	1.476	30
11.	7.688	4.781	2.907	1.645	30
12.	7.294	4.075	3.219	1.266	34
13.	7.813	5.181	2.632	1.969	30
14.	7.125	4.568	2.557	1.730	30
15.	6.906	4.606	2.300	2.000	30
16.	7.813	4.744	3.069	1.546	30
17.	7.938	4.450	3.480	1.250	30
18.	7.688	5.150	2.538	2.029	30
19.	7.875	4.100	3.775	1.086	30
20.	7.438	5.213	2.225	2.342	30
21.	7.750	4.819	2.931	1.644	30
22.	7.875	4.275	3.600	1.188	30
23.	7.494	4.675	2.818	1.659	30
24.	8.138	4.694	3.444	1.363	34
25.	7.038	4.413	2.624	1.682	30
26.	7.918	4.800	3.118	1.219	30
27.	7.565	4.956	2.609	1.900	34
28.	7.875	4.744	3.131	1.515	30
29.	7.625	4.975	2.650	1.877	30
30.	7.906	4.913	2.993	1.642	26
31.	7.250	4.613	2.637	1.749	34
32.	7.250	4.250	3.000	1.417	34
33.	7.563	4.519	3.044	1.484	30
34.	7.938	4.744	3.194	1.485	30
35.	8.063	5.188	2.875	1.805	30
36.	8.000	4.556	3.444	1.323	30
37.	7.750	4.450	3.300	1.348	30
38.	7.188	4.869	2.319	2.099	30
39.	7.500	4.363	3.137	1.391	30
40.	6.600	3.750	2.850	1.319	30
Mean values	7.531	4.579	2.949	1.572	30.4
Stand.dev.	0.369	0.344			

precipitation methods, which give too high albumin values, this perhaps does not present the differences in the fractions of men and women from becoming apparent:

Rinehart (4). Determined with 31 normal women and 30 normal men.

	Tot. Prot.%	Alb.%	Glob.%
Women	6.6—7.3	4.7—5.2	1.5—2.4
mean values	6.94	4.96	1.99
Men	6.9—8.6	4.0—5.8	1.6—3.8
mean values	7.55	5.01	2.47

Salvesen (5). Determined with 16 normal women and 16 normal men.

	Tot. Prot.%	Alb.%	Glob.%	A/G
Women	6.34—7.96	3.77—4.80	2.18—3.55	1.26—2.00
mean values..	7.02	4.35	2.68	1.62
Men	6.53—7.96	3.95—5.24	1.96—3.16	1.43—2.26
mean values ..	7.00	4.44	2.58	1.72

Milam and Durham (2). Investigation is carried out with white and coloured subjects. The values obtained with white people of whom 267 were women and 387 men, are given below.

	Tot. Prot.%	Alb.%	Glob.%	A/G
Women, mean values	7.29	4.61	2.73	1.69
Men, » »	7.12	4.59	2.58	1.79

These results show that the globulin values for women are higher and that the A/G is thus somewhat smaller than for men.

When I compare the results I have obtained in my investigations:

	Tot. Prot. %	Alb. %	Glob. %	A/G
Women, mean values	7.33	4.19	3.32	1.29
Men, » »	7.53	4.58	2.95	1.57

the above theory holds good even by the methanol precipitation method, which is supposed to be the best of all precipitation methods.

SUMMARY

In this investigation the proportion of albumin and globulin fractions of 40 normal men, aged 20—35, have been determined by the methanol precipitation method. A comparison with the

albumin and globulin proportion of normal women determined by the same method shows that it is somewhat higher in men. A reference is also made to the prevalent opinion in the literature that the globulin content of the serum is relatively higher in women than in men.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

SOME HEMAGGLUTINATION TESTS WITH SERA OF PATIENTS SUFFERING FROM RHEUMATOID ARTHRITIS [1]

By

ODD A. WAGER

(Received for publication September 28, 1949)

Agglutination phenomena occurring in rheumatic diseases became the subject of intensive research about 1930, when Cecill, Nicholls *et al.* (1, 4, 5) observed that sera of persons suffering from rheumatoid arthritis were able to agglutinate hemolytic streptococci in high titers. They considered this phenomenon to be entirely specific, a true antigen-antibody reaction, as the Widal test in typhoid fever. — Soon after the publication of the results of the aforementioned investigators, Dawson *et al.* (2, 3) noted that sera of patients suffering from rheumatoid arthritis were without exception and in almost as high titres able to agglutinate equally well the non-encapsulated pneumococci as the hemolytic streptococci. This observation rendered the specificity of streptococcal agglutination in rheumatoid arthritis disputable to some extent. — Oker-Blom (6, 7) recently reported that the sera of patients suffering from rheumatoid arthritis agglutinated several different strains of *Staphylococcus aureus* in almost as high titers as hemolytic streptococci. In addition to this both »streptococcal and staphylococcal agglutinins» were brought to disappear from rheumatoid arthritis sera by absorption with a certain *Staphylococcus aureus* strain.

[1] This work is a preliminary report from a study concerning agglutination conditions in rheumatoid arthritis.

This was not the case when corresponding absorption experiments were made with streptococcus and staphylococcus immune sera. — Olhagen (8) isolated enterococci (*Streptococcus faecalis*) from the pharynx of patients suffering from rheumatoid arthritis and he observed that these enterococci are agglutinated by rheumatoid arthritis sera in higher titers than by other sera. — The investigations of Wallis (11) demonstrated that also collodion particles are agglutinated by rheumatoid arthritis sera. He explained the agglutination of hemolytic streptococci by these sera by the concept that the human nasopharynx serves as a natural »reservoir» for hemolytic streptococci, as a result of which weak streptococcal agglutinins are developed in the serum. Rheumatoid arthritis sera are able in a non-specific manner to intensify the action of these agglutinins, so that they can be distinguished in agglutination tests in vitro. This property of the rheumatoid arthritis sera is related to their ability to agglutinate collodion particles.

Waaler (10) worked earlier along the same lines as Wallis; he demonstrated that the sera of patients suffering from rheumatoid arthritis are able to agglutinate in high titers the red blood cells of sheep, sensitized by a small amount of specific anti sheep cell serum. He ascribes this phenomen to the presence of an agglutination activating factor in rheumatoid arthritis sera which activates the specific immune serum used for sensitizing the sheep red cells so that agglutination occurs in high dilutions, in which the immune serum alone without the presence of rheumatoid arthritis serum is inactive.

Thus both Wallis and Waaler arrive in principle at the same result, i.e., that the sera of persons suffering from rheumatoid arthritis have a non-specific property which intensifies the action of agglutinins. — The phenomenon observed by Waaler has recently become the subject of wider attention through the investigations of Rose *et al.* (9).

In the work described below an attempt was made to clarify the capacity of rheumatoid arthritis sera to intensify specific agglutination. We studied the extent to which the agglutination of the patient's own red cells by a homologous anti-human serum is intensified by the sera of patients with rheumatoid arthritis. On the other hand we tried to find out whether any difference in this respect can be observed between the sera of patients with

rheumatoid arthritis and of healthy persons. Analogous tests have also been made with the red cells of sheep and anti sheep cell serum.

MATERIAL AND METHODS

Sera and red cells from two patients suffering from rheumatoid arthritis and two healthy persons were used. The sera were inactivated by keeping for half an hour in a water bath at 56° C. The red cells were taken into a sodium citrate solution[1] (8 parts whole blood to 1 part citrate solution). The cells were washed three times with saline before use. — The immune sera required for sensitization of the cells were prepared as follows:

Immune serum No. 1: Rabbit No. 1 was immunized with red cells from rheumatoid arthritis patient S.M., belonging to blood group A.

Immune serum No. 2: Rabbit No. 2 was immunized with red cells from rheumatoid arthritis patient I.H., belonging to blood group 0.

Immune serum No. 3: Rabbit No. 3 was immunized with red cells from healthy person O. W., belonging to blood group 0.

Immune serum No. 4: Rabbit No. 4 was immunized with red cells from healthy person H. N., belonging to blood group A.

The rabbits were immunized by intravenous injection of a 5 per cent red cell suspension in saline at intervals of 5 days in doses of 0.5, 1.0 and 2.0 ml. The rabbit immune sera obtained in this manner were inactivated by keeping for one-half hour in a water bath at 56° C.

Sensitization of Red Cells. — In each case the cells were sensitized with the homologous immune serum. Thus the cells of patient S.M. were sensitized with immune serum No. 1, the cells of patient I.H. with immune serum No. 2, etc. This was done by adding to the 2 per cent red cell suspension in saline an equal volume of the corresponding immune serum dilution (½ minimum agglutinating dose). This suspension was prepared immediately before the use of the cells for the main agglutination test. The

[1] Sodium citrate 2.67
Glucose 2.2
Citric acid 0.9
Aq. dest. ad 100.0

determination of the ½ minimum agglutinating dose was always performed before the sensitization of the cells, by the following method:

An immune serum dilution series of 1: 200, 1: 400, 1: 600, 1: 800, etc. was made using 0.5 ml in each tube; 0.5 ml of 2 per cent red cell suspension in saline and 1.0 ml of saline solution were added. The tube series was kept for one hour in a water bath at 37° C and overnight in the refrigerator. The agglutinations were read by inverting each tube twice and looking with the naked eye against a lamp. If the highest serum dilution in which agglutination was observed was, e.g., 1: 1000, the dilution chosen for the main test was 1: 2000 (= ½ minimum agglutinating dose) which was added to the 2 per cent red cell suspension in sensitizing the cells.

The agglutination test (the main test) with patient serum and the sensitized red cells of the same person was carried out as follows:

A dilution series of 1: 1, 1: 2, 1: 4, 1: 8, etc. was made of the patient serum, using 0.5 ml in each tube, and 0.5 ml of the sensitized red cell suspension of the same patient were added. Thus the actual dilutions of the patient serum were 1: 2, 1: 4, 1: 8, etc. Two tubes were used as controls, one containing 0.5 ml saline solution and 0.5 ml sensitized cell suspension, the other 0.5 ml patient serum 1: 1 and 0.5 ml non-sensitized 1 per cent cell suspension. The tube series was held in a 37° C water bath for one hour and in a refrigerator overnight, and readings were taken as explained in connection with the titration of the immune serum.

RESULTS

The results obtained are given in Table 1. In all the four cases the investigated serum agglutinated its own cells sensitized by rabbit immune serum prepared with them. In both cases of rheumatoid arthritis this agglutination extended to considerably higher serum dilutions (S.M. ad 1: 1024, I.H. ad 1: 512) than was the case with the healthy persons (O.W. ad 1: 2, H.N. ad 1: 8). The controls revealed that the immune sera alone in the used ½ minimum agglutinating dose without the presence of patient serum did not produce agglutination, nor did the patient sera alone without the presence of immune serum agglutinate their own cells.

In further agglutination tests with the serum of rheumatoid

TABLE 1

AGGLUTINATION OF THE PATIENT'S OWN SENSITIZED RED CELLS BY THE PATIENT'S OWN SERUM IN TWO CASES OF RHEUMATOID ARTHRITIS (S.M. AND I.H.) AND TWO NORMAL CASES (O.W. AND H.N.)

		Serum Dilution												
		1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096	Saline Control
Case S.M.	NS	−												
	SS	+++	++	++	++	++	++	++	++	+	+	−	−	−
Case I.H.	NS	−												
	SS	++	++	++	++	++	+	+	+	+	−	−	−	−
Case O.W.	NS	−												
	SS	+	−	−	−	−	−	−	−	−	−	−	−	−
Case H.N.	NS	−												
	SS	+	+	+	−	−	−	−	−	−	−	−	−	−

NS = Patient's own non-sensitized red cells.
SS = Patient's own sensitized red cells.

Degree of Agglutination:

++++ = Red cells form one solid cake, which remains mainly intact after inversion of tube.

+++ = After inversion of tube, red cell cake breaks up into several parts; liquid clear.

++ = After inversion of tube, red cells form numerous agglutinated clumps of various size; liquid cloudy.

\+ = After inversion of tube, red cells form small, equal-sized agglutinated bodies, clearly discernible macroscopically.

arthritis patient S.M. and the sensitized red cells of healthy person O.W., the results were exactly analogous to those obtained with S.M.'s own sensitized red cells. S.M. belonged to blood group A and O.W. to blood group 0. Neither did there seem to be any difference which of the four immune sera (Nos. 1—4) were used for sensitizing the cells.

Parallel with the above described tests, analogous agglutination tests were made with the same patient sera, using adequately sensitized sheep red cells, and employing mainly the technique of Rose *et al.* (9). However, the titration of immune sera was performed in the manner explained above by determining the agglutinin titer of the immune sera, and not by basing it on the titration of the hemolysin used by Rose *et al.* The results are given in Table 2:

TABLE 2

AGGLUTINATION OF SENSITIZED SHEEP RED CELLS BY THE SERA OF TWO PATIENTS WITH RHEUMATOID ARTHRITIS (S.M. AND I.H.) AND TWO HEALTHY PERSONS (O.W. AND H.N.)

		Serum Dilution										
		1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048	1/4096	1/8192	Saline Control
Case S.M.	NS	+++	+	−	−	−	−	−	−	−	−	
	SS	++++	++++	++++	+++	+++	+++	+++	++	+	−	−
Case I.H.	NS	+++	++	−	−	−	−	−	−	−	−	
	SS	+++	+++	+++	+++	++	++	+	−	−	−	−
Case O.W.	NS	++	−	−	−	−	−	−	−	−	−	
	SS	+++	++	−	−	−	−	−	−	−	−	−
Case H.N.	NS	+++	++	−	−	−	−	−	−	−	−	
	SS	++++	+++	+++	+	−	−	−	−	−	−	−

NS = Non-sensitized sheep red cells.
SS = Sensitized sheep red cells.

Accordingly both rheumatoid arthritis sera agglutinated the sensitized sheep cells in considerably higher serum dilutions than the sera of the healthy persons did (S.M. ad 1: 4096, I.H. ad 1: 1024, O.W. ad 1: 32 and H.N. ad 1: 128). In contrast to the human cell agglutination test, also non-sensitized sheep cells were agglutinated. This agglutination did not, however, in any case reach higher serum dilutions than 1: 32, and no significant difference was in this respect discernible between the sera of patients with rheumatoid arthritis and healthy persons. Immune sera without the presence of patient sera did not produce agglutination in the dilutions used.

DISCUSSION

No definite general conclusions can be drawn from the results obtained, in view of the small series of cases studied. But it is rather interesting to see that the red cells of the two patients with rheumatoid arthritis, which were sensitized with homologous rabbit immune serum, were agglutinated by the patients' own sera in considerably higher serum dilutions than the correspondingly treated red cells of the two healthy persons were agglutinated by

the latter's own sera, and that the same phenomenon was seen also in using sheep red cells sensitized with homologous rabbit immune serum. — It seems probable that the same factor in the rheumatoid arthritis serum which causes the agglutination of the patient's own sensitized red cells in high serum titers also causes the sensitized sheep red cells to agglutinate in considerably higher serum titers than non-sensitized sheep red cells. Is the question in both cases one of a quantitative increase in a property already existing in the normal serum? The studied normal sera to some extent agglutinated the patient's own sensitized red cells and to a greater extent agglutinated sensitized than non-sensitized sheep red cells. — In looking for the reason why rheumatoid arthritis sera so strongly agglutinate sensitized red cells, two alternatives present themselves:

1. The effect of the heterophilic agglutinins in the patient serum has become better visible by using a very sensitive technique. This hypothesis is less probable because also the patient's own red cells which have been sensitized by homologous immune serum are agglutinated by the patient's own serum.

2. There is a non-specific factor in the patient serum which activates the agglutination of red cells by homologous immune serum. This probably is the case. In particular the agglutination of the patient's own sensitized red cells by the patient's own serum strongly supports this assumption.

The surprising agglutination phenomena observed in rheumatoid arthritis sera may possibly be explained by such a non-specific agglutination activating factor. For instance, the same non-specific agglutination activating factor which acts in the agglutination of red cells by homologous immune serum, may activate the agglutination between certain bacteria vegetating in the human body (streptococci, pneumococci, staphylococci and enterococci) and the weak agglutinins created by them which already normally are present in the human serum. This conclusion is the same as that reached by Wallis (11) in his attempt to explain on the basis of the agglutination of collodion particles the agglutination phenomenon of hemolytic streptococci in rheumatoid arthritis.

SUMMARY

The capacity of the sera of two patients suffering from rheumatoid arthritis and two healthy persons to agglutinate red cells sensitized with specific homologous immune serum was studied. It was found that the sera of patients suffering from rheumatoid arthritis effect a stronger agglutination of the patient's own sensitized red cells as well as of sensitized sheep red cells than do the sera of healthy persons. — The conception brought forth by earlier investigators that a non-specific agglutination activating factor is present in the sera of patients with rheumatoid arthritis is discussed. The results obtained in this work seem to support the opinion that such a factor exists.

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FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI

STUDIES ON THE NATURE OF HEMAGGLUTININS PRESENT IN SEEDS

By

K. O. RENKONEN

(Received for publication November 1, 1949)

Our knowledge of the nature of hemagglutinins present especially in the family of *Papilionaceae* is based principally on the studies of the bean and Jack bean agglutinins. The former have been studied, among others, by Wienhaus (7) and Schneider (5). Wienhaus considered his purified »Phasin» not a true protein, while Schneider speaks of proteose. Eisler and Portheim (2) think that the agglutinins do not belong to proteins but are conjugated with them. On the other hand, the Jack bean agglutinins have been crystallized by Sumner and Howell (6) as »concanavalin», a globulin. Boyd and Reguera (1) seem to have isolated from Lima beans the anti-A specific agglutinins as globulins.

We have studied[1] the agglutinins present in beans and in seeds of *Vicia Cracca*. From a saline extract of bean flower (1: 10) some rather inactive components were precipitated with 25 per cent by vol. ethyl alcohol at room temperature. The active components were then precipitated from the supernatants (2 parts) with acetone (1 part). The precipitate dissolved in saline was shaken with chloroform, centrifuged, and reprecipitated with acetone *ana partes*.

[1] For technical assistance I wish to express my sincere thanks to Miss Brita Schulman.

The procedure was repeated twice after a successive reduction of the bulk to 1/10 of original volume. The purified preparation generally had a titre of the order of 1: 100,000 measured with human red cells. This is probably not very far from the purified fractions of earlier investigators. An effective further purification does not seem to be easy to carry. The fractionated precipitation with acetone from saline yielded first some fractions containing less carbohydrates [1] and in the end some fractions containing more than did the middle fractions. The middle fractions were the most active ones, but the difference between them and the original preparation was slight, some ten per cent, and was not always distinctly measurable.

The elementary analysis of the purified preparation gave the following records:

Signum	3/8/48	26/4/49	18/1/49
C	36.65%	41.79%	
H	6.48	7.44	
N	9.7	9.0	
Ashes	13.26	5.69	
P		0.096	
S			0.49%

The product contained about 30 per cent carbohydrates. The Biuret test was strongly positive, indicating that the remainder of the preparation was related to proteins. The nature of the preparation, however, was not that of proteins, for trichloracetic acid (10 per cent), for instance, did not precipitate a one per cent solution, while tannic acid did so immediately.[2] The minimum of solubility of our water-soluble preparation occurred between pH2 and 3.

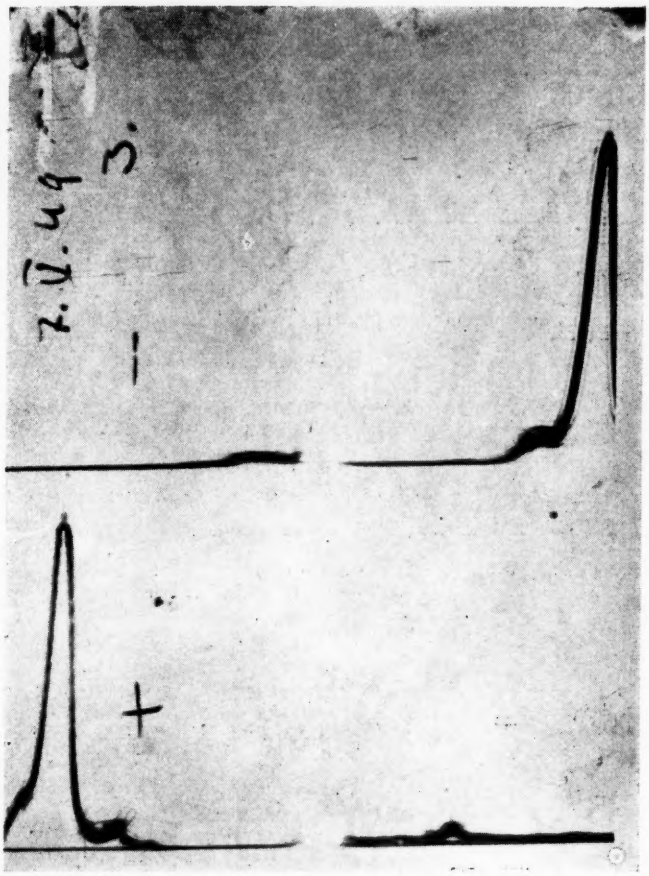
Electrophoretical analysis [3] gave the following picture: —

---

[1] Measured by the orcin test according to Rimington (3) (dextrin standard).

[2] It was possible to make the same active preparation from the supernatants of the first bean extract after a precipitation of ordinary proteins with trichloracetic acid.

[3] My sincere thanks are due to Mr A. Louhivuori for the electrophoretic analysis of our products.



The activity was combined with the slow big peak. At further running, this was split into two equal peaks, but when separating them no purification could be obtained. Both fractions corresponding to these peaks were equally active.

When we treated the seeds of *Vicia Cracca* (4) in the same manner we obtained a preparation with titre up to 1/300,000 measured with A1 cells. It contained 10 per cent of nitrogen and 30 per cent of carbohydrates. It gave electrophoretical pictures similar to those of the bean preparations and preserved the specific anti-A1 agglutinins.

In order to evaluate the purity of our *Vicia Cracca* preparation we absorbed a solution of it with A1 and B cells and compared the

TABLE

CARBOHYDRATE CONCENTRATION OF VICIA CRACCA AGGLUTININS

Date of Experiment	Titre of Unabsorbed Agglutinin Solution	Absorption with A1 Cells		Absorption
		Titre of Agglutinin Solution	% of Diminution of Agglutinins	Titre of Agglutinin Solution
13/10/49	1/22	1/2	ca. 90	1/18
17/10/49	1/40	1/2,2	ca. 95	1/32

The agglutinins were measured with A1 cells.

TABLE

AGGLUTINATION INHIBITION TESTS OF ANTI-VICIA SERA NO. 26, 27, 28, OF COR-IMMUNISATION

Dilution of Agglutinating Extracts or sera	Agglutinins of Vicia Cracca (Starting with Dilution 1/500) Measured with A1 and B Cells													
	26		26N		27		27N		28		28N		NaCl	
	A1	B	A1	B	A1	B	A1	B	A1	B	A1	B	A1	B
1/1	++	++	++	++	+	+	++	+	±	±	++	+	++	+
1/2	++	+	++	+	+	+	++	+	−?	±	++	+	++	+
1/4	++	+	++	+	+	+?	++	+?	−?	±	++	+?	++	+
1/8	++	−?	++	−?	+	−	++	+?	−?	−	++	±	++	+?
1/16	+	−	++	−	−	−	++	−	−	−	++	−?	++	−?
1/32	−?	−	++	−	−	−	++	−	−	−	++	−	+	−
1/64	−	−	++	−	−	−	++	−	−	−	++	−	+	−
/128	−	−	++	−	−	−	++	−	−	−	++	−	++	−
1/256	−	−	+	−	−	−	++	−	−	−	++	−	++	−
1/512	−	−	++	−	−	−	++	−	−	−	+	−	+	−
1/1024	−	−	−?	−	−	−	+?	−	−	−	±	−	+?	−
1/2048	−	−	−	−	−	−	−	−	−	−	−	−	−	−
1NaCl	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[1] The volume of agglutinating extract and of human serum dilutions was 0.1 ml. The inhibiting anti-Vicia sera and their control sera were in 1/10 dilution and in 0.1 ml volume. The volume of the 3% blood suspension in saline was 0.1 ml. Before the addition of red cells the stands were kept for one hour at 37° C. The agglutination results were read by the naked eye after one more hour, during which the stands were kept at room temperature. The signs + and ++ indicate positive and strongly positive reactions. The sign − indicates a negative and signs +? ± and −? different degrees of doubtful reactions.

1

BEFORE AND AFTER ABSORPTION WITH A1 AND B CELLS

with B Cells	Carbohydrate Concentrations		
% of Diminution of Agglutinins	Control before Absorption	After Absorption in % of that of the Control	
		Absorption with A1 Cells	Absorption with B Cells
20	0.032 mg/ml	95	118
20	0.032 mg/ml	85	129

Dilution of the Vicia Cracca product was 1/10,000 before the absorption. The absorption was performed in icebox with 10 % of washed cells. The time for absorption was two hours.

2

RESPONDING CONTROL SERA NO 26N, 27N, 28N FROM THE SAME RABBITS BEFORE AND OF SALINE

Agglutinins of Human B and A Sera (Starting with Dilution 1/1) Measured with A1 and B Cells												
26		26N		27		27N		28		28N		NaCl	
A1	B	A1	B	A1	B	A1	B	A1	B	A1	B	A1	B
++	++	++	++	++	++	++	++	++	++	++	++	++	++
++	++	++	++	++	++	++	++	++	+	++	++	++	++
++	+	++	++	++	++	++	++	++	+	++	++	++	++
++	+?	++	++	++	+?	++	+	++	±	++	+?	+	+?
+	±	++	+?	++	−?	++	±	++	−?	++	−?	+	−?
+	−?	+	−	++	−	++	−	++	−	++	−	+?	−
±	−	−?	−	+?	−	−?	−	+	−	−?	−	−?	−
±	−	−	−	−?	−	−?	−	+?	−	±	−	−	−

carbohydrate content in both supernatants and in the non-absorbed control (table I). Since slight hemolysis sometimes occurs after absorption (cf. the experiment 17/10/49), we measured the degree of hemolysis, and a carbohydrate analysis from a control of equally hemolysed (with water) red cell solution was performed and this value subtracted from the carbohydrate value of the supernatant. — Unfortunately an analogous analysis of the nitrogen content instead of carbohydrates was not so reliable because of the high nitrogen values of the hemolysed controls.

Table 1 shows that there was a slight decrease in the amount of carbohydrates after the absorption with A1 cells. This supernatant contained distinctly less carbohydrates than the supernatant after a B cell absorption. The latter contained more carbohydrates than the unabsorbed control, due to the traces of carbohydrates present also in a blank non-hemolysed absorption control test (saline + 10 per cent washed cells).

The interpretation of these experiments invites the suggestion that the agglutinins in *Vicia Cracca* contain a carbohydrate component, but our product seems still to be crude, containing perhaps some 20—30 per cent of the active substance which ought to have a titre presumably of 1/1,500,000.

In order to obtain an idea of its antigenic nature and to compare this anti-A1 agglutinin with human anti-A agglutinin we immunised three rabbits with 5 mg of the purified Vicia preparation twice a week, six times in all. After this all the rabbit sera contained strong anti-Vicia antibodies as measured by complement fixation test.

The agglutination inhibition test was performed with these sera and with the corresponding control sera (table 2). Evidently no inhibition of agglutinins of the *Vicia Cracca* preparation was caused by the control sera, but a pronounced inhibition of the anti-A agglutinins of Vicia was exerted by all the immune sera. Consequently there is some reason to suppose that the anti-A agglutinin of *Vicia Cracca* is antigenic. If we assume that the entire inhibition was due to the general anti-Vicia antibodies, it is difficult to explain why the anti-B agglutination was not equally affected.

The anti-Vicia sera did not affect the human isoagglutination reactions. Accordingly the *Vicia Cracca* anti-A agglutinin seems to differ from the human anti-A agglutinin.

DISCUSSION AND SUMMARY

An attempt was made to purify the agglutinating substances isolated from the bean and *Vicia Cracca*. This was done by methods favorising the purification of mucoproteins. Accordingly the agglutinating purified preparations behave in many respects like mucoproteins. The agglutinin content of the purified *Vicia Cracca* preparation was roughly evaluated at some 20 per cent on the basis of a decrease in the carbohydrate content after a specific agglutinin absorption. In immunological tests some evidence was found that the Vicia anti-A is antigenic and differs from the human anti-A.

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Aided by a grant from Emil Aaltonen Foundation.

FROM THE INSTITUTE OF PATHOLOGICAL ANATOMY, UNIVERSITY OF HELSINKI

AN ACCURATE AND QUICK METHOD FOR INTRAGASTRIC ADMINISTRATION OF SOLUTIONS (CARCINOGENIC HYDROCARBONS) TO MICE

By

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(Received for publication November 2, 1949)

Carcinogenic hydrocarbons have been generally administered by the mouth either with food or as a drink. If the carcinogen was introduced as a drink, is was given either instead of drinking water from wide-mouthed bottles closed by a rubber stopper, into which a glass tube was inserted with an opening of a few millimeters at the top (2), or the aqueous suspension was added to the milk given to the mice who had been without food for 12 hours (1). The carcinogen was administered with the food either by allowing single drops of the oily solutions to soak into small pieces of rat cake or toasted bread (3, 1), or the hydrocarbon was added to the dietary mixture by dissolving it in melted Crisco (5).

These methods, however, have considerable limitations. Unsavoury substances were left uneaten by the mice. Losses from spilling, even if inconsiderable, must yet be taken into account. The daily intake of an individual mouse has generally been deduced from the quantity consumed by the whole series during the experiment. Yet it is natural that the daily dose ingested by different mice varies greatly, as well as the doses consumed by the animals on different days.

In order to counteract these evident drawbacks, we have developed a method which has proved serviceable, and whose publication therefore appears justified.

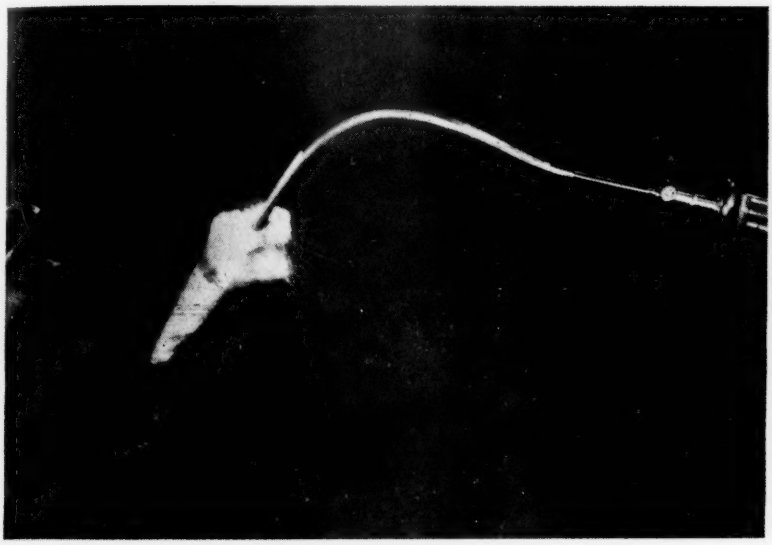

Fig. 1. — Photograph illustrating how the feeding is performed.

The solution is introduced direct into the forestomach of the mouse by means of a tube, one end of which is connected with a hypodermic syringe. For tubing we have used a polythene cannula (Allen & Hanburys Ltd.) with a diameter of 1 mm. To prevent the mice from gnawing the cannula, it was inserted into a slightly wider tube about 2 cm long, which the mice were allowed to chew and through which the thin tube already referred to passed without any difficulties. As compared to a gag, this arrangement has the advantage that the cannula is easier to insert into the esophagus if the animals are allowed to gnaw. It is moreover easier to handle a tube than a gag, since it remains fixed on the cannula. Before use the end of the cannula must be heated in hot water, to render it more pliable. Before the transfusion the animal must be laid on its back, its forefeet being firmly fixed on the surface of the table by pressure with the first and second finger of the left hand (fig. 1). The tube is fitted with a mark at about 5.5 cm from one end; when it is flush with the incisors, the other end has reached the forestomach, and the substance in question can thus be injected direct into the stomach.

We have been using this method for about 8 months, and it has

proved serviceable in every respect.[1] The animals have borne even a daily transfusion well, although we have used by preference a transfusion of 2—3 times a week. The method is also comparatively quick, since depending on the degree of practice, one can handle in this way 100 to 150 animals per hour. A drawback of the method is perhaps constituted by a continual slight trauma or irritation due to it, although we have so far been unable to find any definite signs of it. On the other hand, the benefits derived from the method are: accurate dosage; the substance required can be administered at the desired moment; gastric aspiration can be easily performed. In addition, the method is simple and comparatively quick.

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[1] Squamous cell carcinomata of the forestomach have appeared after 3 months, during with time not more than 1.65 mg of carcinogenic hydrocarbon were ingested (4).

FROM THE DEPARTMENT OF SEROLOGY AND BACTERIOLOGY, UNIVERSITY OF HELSINKI, AND FROM THE STATE SERUM INSTITUTE, HELSINKI

CATGUT TETANUS

By

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(Received for publication November 15, 1949)

The infection mechanism of postoperative tetanus is of a complex nature. The fact that tetanus bacilli or their spores are present in the human intestinal system in 40 per cent (3) or 34.7 per cent (39) of people makes the intestines a possible source of infection in the cases where they have been opened in the course of the operation (37, 43 *et al.*). In operations of the pelvic region the infection may be caused by contamination by feces on the skin (24). In certain cases tetanus has appeared after aseptic foot and sole operations on patients who had the habit of walking barefoot (19, 38). It has been possible to demonstrate tetanus bacilli in samples taken from both the feet and the hair (41). According to Schmid (35) the presence of tetanus bacilli on the skin is so common that he regards the infection simply as a manual transference of microbes from the skin into the deeper tissues during the operation. In certain cases the infection may probably have been conveyed by medical injections, such as of gelatin (14, 25, 42 *et al.*), diphtheria serum (36) and quinine (7, 22, 40) or by vaccination (12 and 20). The instruments, gloves and even the dressings (13) used during the operation have been considered probable sources of infection. In other cases the possibility of infection from the bandages used before and after the operation or from their cotton raw material could be demonstrated (4, 10, 23 and 31).

Special attention has been paid to the suture remaining in the tissue. The sterilisation of silk usually presents no difficulties and

it cannot be regarded as a particular source of infection, but the case is different with catgut. The sheep intestine used as raw material contains a large amount of spores of anaerobic bacteria which are extremely difficult to destroy. Thus Piening (30) demonstrated the presence of tetanus bacilli in 25 per cent and of gas gangrene bacilli in 100 per cent (*Cl. welchii*, 100 per cent; *Cl. novyi*, 80 per cent; *Cl. septicum*, 15 per cent; *Cl. histolyticum*, 5 per cent) of dried Spanish, English and Argentine sheep intestines. For this reason it is important to clarify the significance of catgut as a probable source of infection. I have found over 400 reported cases of postoperative tetanus, in the majority of which catgut was used in the surgical operation. Catgut was suspected of being the source of infection in a great number of these cases, and numerous bacteriological examinations were carried out to ascertain the presence of tetanus bacilli, but success was achieved in very few cases only.

Thus Jaffe (16) was able to isolate from catgut in one case bacteria which morphologically resembled tetanus bacilli, but he was not allowed to make the final identification by means of an animal test. (The author adds: »This is a triumph for antivivisectionists».) Dyke (8) reported a case where the bacilli were identified by animal experiments. Nicholle (29) demonstrated the presence of tetanus bacilli by implanting in the test animal a piece of the same catgut as was used in the operation. Koch (18) demonstrated tetanus bacilli by animal tests with a sample of catgut taken from the patient at autopsy. Only Murstad (28) and Mackie (21) had several cases. The former was able to demonstrate the presence of tetanus bacilli in four cases, while the latter listed nine postoperative cases of tetanus, in two of which he was able to establish that catgut was the source of the tetanus bacilli. In addition to this, bacteria resembling tetanus bacilli developed from two catgut samples. Richardson (32) mentions in his report four cases in which bacteriological investigations of the catgut revealed bacteria resembling tetanus bacilli but animal tests were negative. Four postoperative cases of tetanus in New South Wales in which tetanus bacilli were obtained from catgut cultures have been reported (J.A.M.A. 1934:*102*:2:1172).

Although the present writer has not been able to obtain by far all the reports on postoperative tetanus cases, even those

mentioned above indicate that it has been possible to find tetanus bacilli in catgut in very rare cases only. In view of this it is not surprising that the role of catgut in the infection mechanism of postoperative tetanus seems to be considered more of theoretical than of practical significance. Bulloch (2), for example, calls catgut tetanus a bogy, more talked about than it would deserve. He reports having investigated himself 17,420 samples of catgut without finding tetanus bacilli in a single one of them.

Against this background it seems fully justified to review the thirteen cases of postoperative tetanus which occurred in Finland during the years 1945—1947 and in which infection from catgut was suspected. The catgut used in all these cases had been prepared by the same manufacturer and was reported by him to be chemically sterilised. Unfortunately, samples of catgut were sent for examination in nine cases only, in addition to pieces of tissue removed at autopsy in three cases. Part of the catgut samples were in opened and partly used packages and a part in the factory's original unopened cartons. Five of the cases of postoperative tetanus have been earlier mentioned briefly in another connection (33), but since in the course of continued investigations essentially new aspects have appeared in three of these cases, they have been included among the nine above-mentioned cases.

The catgut examinations were made by the method described in principle by Meleney and Chatfield (26) and modified to some extent by Clock (6), Brewer (1) and Christensen (5). A special room was reserved for the investigations, in which the bacterial content of the dust was controlled during the handling of the catgut by uncovered blood agar and glucose blood agar dishes, which were incubated in a thermostat at 37°C for 24 to 48 hours, in addition to which the glucose blood agar dishes were kept in a Zeissler (44) vacuum exsiccator. An identical number of covered blood agar and glucose blood agar dishes were treated at the same time in a similar manner to prove that the culture media as such did not contain living bacteria.

The catgut samples were handled with long forceps and long scissors burned in a Bunsen flame and the catgut was cut into lengths of about two centimetres directly into large sterile test tubes closed with cotton wadding. The unopened packages were opened in as sterile a manner as possible using the same instru-

ments. Sterile neutralisation liquid, consisting of 1 per cent sodium thiosulphate and 1 per cent sodium carbonate dissolved in distilled water was added to the tubes, which then were incubated for 24 hours in a thermostat at 37° C, after which the neutralisation liquid was replaced by distilled water and the incubation repeated as above. The pieces of catgut were thereupon transferred in a sterile manner with forceps into the culture medium tubes, approximately one-half lineal metre into each tube, and the tubes were placed into a thermostat at 37° C. The control time was 21 days in accordance with Christensen's method (5). Observations of possible growth were made at 1, 3, 7, 14 and 21 days.

Liver broth and soft glucose agar (34) as culture media were used. After preparation they were sterilised in an autoclave and immediately before the culture kept for at least 10 minutes in a water bath at 100° C to remove the oxygen dissolved in the medium. After the culture media, which had a pH between 7.2 and 7.4 had cooled or been cooled to a temperature of 45° C or below, the catgut pieces were transferred into the media. No seal was used on the surface of the culture media.

The object of the bacteriological investigation was chiefly the observation of spore-forming anaerobic bacteria. The effort was to isolate the anaerobes as growth occurred, using, in addition to the test tube culture media mentioned above, glucose blood agar dishes as described by Zeissler (44). In accordance with this method the cultures were heated to 80—100° C for periods of varying length, after which subcultures were made from the heated cultures as required into either the glucose blood agar dishes or the soft glucose agar or liver broth tubes. The glucose blood agar dishes were incubated for 3 days in a thermostat at 37° C in Zeissler vacuum exsiccators and the vacuum was controlled daily by a manometer. The isolation of strains of tetanus bacilli was in particular a very difficult task, entailing great patience and much work.

The identification of the strains was performed chiefly in the manner described by Zeissler (44), taking into consideration the morphological properties of the bacteria and their manner of growth on glucose blood agar dishes as well as the fermentative capacity of pure cultures. The last mentioned were studied by using broth to which had been added 1 per cent of the following

carbohydrates (prepared according to Zeissler): glucose, lactose, maltose, mannite, starch, salicin, insulin, galactose, glycerin and levulose, as well as milkliver and brain broth culture media in tubes. Animal tests were performed only for the identification of tetanus bacilli.

In addition to tests on the bacterial content of the air-borne dust, the following control cultures were made: from the distilled water and the neutralisation liquid into at least two culture medium tubes each, from each catgut examination series into at least. two culture medium tubes. Growth control was made using *Cl. bifermentans*. Physiological saline dilutions of 1: 1,000 and 1: 1,000,000 were made from a 24-hour-old liver broth culture of this strain and two normal loopfuls of each dilution were inoculated into tubes containing soft glucose agar and liver broth. All control cultures were incubated in a thermostat at 37° C for 21 days, Growth in both of the growth control dilutions was considered an indication of the efficiency of the culture media.

Table 1 shows the diagnoses, operations performed, incubation time for tetanus, results of treatment, and bacteriological examinations made, including animal tests, in each of the cases of postoperative tetanus.

A study of the diagnoses reveals that abdominal operations in which the intestinal system was opened were performed on five of the thirteen patients (cases No. 1/45, 2/45, 4/47, 8/47, 10/47) Most of the previously occurred postoperative tetanus cases had generally occurred after an abdominal operation. Many investigators (15, 32, 37 and 43) have pointed this out. Thus Wohlgemuth (43) considers the administration of tetanus antitoxin definitely indicated in all cases where the intestinal system is seriously damaged by operation, and he, as well as Matas (24), demands preoperative intestinal evacuation and diet in cases involving abdominal operation. In general the incubation time for tetanus varied between five and twelve days, after which the case ended fatally within one to two days, except in case No. 3/46 where death occurred within eight days. It deserves to be noted of the recovered cases (No. 9/47, 12/47, 13/47) the incubation time in two (9/47 and 13/47) was as long as thirteen and sixteen days, respectively.

Tetanus antitoxins and narcotics were used for treatment of

TABLE

CASES OF POSTOPERATIVE TETANUS

No. Year	Diagnosis	Operation	Incubation Period	Result
1/45	Ulcus duodeni Tetanus postoperativa	Resect. ventric. Billroth II	10 days	Exitus 2 days
2/45	Myomata uteri Salpingo-oophoritis chr. Tetanus postoperativa	Amput. uteri supra-vagin. Salpingo-oophorectomia Appendicect.	5 »	» 2 »
3/46	Thyreotoxicosis Tetanus postoperativa	Strumectomia	7 »	» 8 »
4/47	Appendicitis ac. gangraen. Peritonitis diffusa inc. Tetanus postoperativa	Appendicect.	11 »	» 1 »
5/47	Myomata uteri submucosa Tetanus postoperativa	Amput. uteri	8 »	» 1 »
6/47	Myomata uteri submucosa Tetanus postoperativa	Amput. uteri supra-vagin.	10 »	» 2 »
7/47	Hernia inguinalis Tetanus postoperativa	Oper. rad. a.m. Bassini	6 »	» 1 »
8/47	Placenta praevia Positio transversalis Tetanus postoperativa	Sectio Caesar. Appendicect.	9 »	» 1 »
9/47	Myomata uteri Tetanus postoperativa	Amput. uteri	16 »	Recovered

1

AND BACTERIOLOGICAL EXAMINATIONS

Wound Infection	Bacteriological Test							
	On Catgut						On Tissue	Pathogenic to Animal
	Samples	Opened	Original	Sterile	Unsterile	Findings		
+	7	7	—	2	5	Cl. tetani? Cl. welchii Cl. multif.	Cl. tetani? Cl. welchii E. coli Streptoc. virid.	
	4	4	—	3	1	Cl. welchii	Streptoc. anaerob. Staph. aur. E. coli	
	2	—	2	—	2	Cl. tetani Cl. welchii Cl. novyi Cl. biferm. Cl. sphenoid.		Mouse +
+ +	8	8	—	3	5	Cl. tetani Cl. novyi Cl. multif. Cl. sphenoid.		Mouse +
	5	5	—	4	1	Cl.tetani Cl. welchii Cl. biferm. Cl. novyi	 Staph. aur.	Mouse + Guinea Pig +
	4	4	—	1	3	Cl. tetani Cl. novyi Cl. histolyt. Cl. biferm. Cl. tertium	 Cl. tetani Cl. novyi Staph. aur.	Mouse + Mouse +
	7	—	7	—	7	Cl. tetani? Cl. novyi Cl. tetani Cl. novyi Cl. biferm.		Mouse +

No. Year	Diagnosis	Operation	Incubation Period	Result
10/47	Myomata uteri permagna Degeneratio cystica ovarii Appendicitis chr. Tetanus postoperativa	Amput. uteri supravaginal. et salpingo-oophorectomia Punctio ovarii Appendicect.	12 days	Exitus 2 days
11/47	Myomata uteri Salpingo-oophoritis chr. Tetanus postoperativa	Amput. uteri totalis Salpingo-oophorectomia	11 »	» 2 »
12/47	Graviditas mensis II—III Tub. pulm. Tetanus postoperativa	Sectio minor Ligatura tubarum	9 »	Recovered
13/47	Thyreotoxicosis Tetanus postoperativa	Strumectomia	13 »	»

the patients described in the table. The mortality rate corresponds with the general rate presented in the literature for tetanus. Unfortunately, no samples of catgut were obtainable for bacteriological examination for the last four cases (No. 10/47, 11/47, 12/47, 13/47). In one case the catgut supply was exhausted. As the catgut used also in these cases had been manufactured by the same factory and as catgut infection was specifically suspected, it seems appropriate to include these cases in the present series.

Wound infection was observed at least in cases No. 1/45, 4/47 and 5/47 but it was fairly mild.

RESULTS OF THE BACTERIOLOGICAL INVESTIGATION

In the first case which occurred (No. 1/45), Gram positive, anaerobic, spore-bearing rod bacteria morphologically resembling tetanus bacilli were found in the catgut. The spores were considerably thicker than the rods, being fully terminal and round, and they were formed within 72 hours. It was not, however, possible to isolate a pure strain and definitely to identify it. It is never-

Wound Infection	Bacteriological Test							
	On Catgut						On Tissue	Pathogenic to Animal
	Samples	Opened	Original	Sterile	Unsterile	Findings		

theless to be noted that morphologically exactly similar anaerobes were found in a tissue sample consisting of the operation wound excised at autopsy.

In the second case, (No. 2/45), no anaerobes other than *Cl. welchii* were found in the catgut and no others from the piece of tissue sample than *Streptococcus anaerobicus.*

In the third case, (No. 3/46), *Cl. welchii* only were obtained from the catgut in the first investigation. In subsequently continued investigations, on the other hand, a strain exactly resembling morphologically and culturally the tetanus bacilli was isolated. Mouse tests, with two pairs of white mice, were performed for its definite identification and for determination of its pathogenicity to animals. All the mice were given 0.2 cc of 48-hour old liver broth culture of the investigated strain, and the control mice (two mice) furthermore 0.2 cc or 200 units of tetanus antitoxin (Statens Seruminstitut, Copenhagen). In these as well as in all later mouse tests the injections were administered intraperitoneally and the liver broth cultures of varying age of the strains under investigation were used either as such or diluted with physiological saline solution.

Both test mice died two days after the injection, whereas the control mice were still lively 30 days after the injection.

The two following cases (Nos. 4/47 and 5/47) occurred almost simultaneously at the same hospital. *Cl. tetani* was found from the thickest (thickness No. 5) of the catgut samples sent for investigation, which had been used in both operations and which the surgeon suspected most of being the source of infection. An animal test with one pair of mice was carried out with pure cultures of this strain in the manner described above, using a 48-hour old culture, of which 0.1 cc was administered to each mouse. One of them died within eight days, whereas the other, which had been given antitoxin (0.2 cc or 200 units) remained lively for 30 days. The test was thereupon repeated with a fresh pair of mice, using again a 48-hour old pure culture. The test mouse died within less than three days, whereas the control mouse remained fully lively for 30 days.

Also in the sixth case (No. 6/47) *Cl. tetani* was found in the thickest catgut (thickness No. 5). A mixed culture was first obtained containing, in addition to rods which morphologically resembled the tetanus bacillus, also anaerobes of the *Cl. bifermentans* type. A guinea pig test was made with a 72-hour old mixed culture grown in liver broth by intraperitoneal administration of 0.5 cc of the culture to one guinea pig, and to the other of 1.0 cc or 1,000 units of tetanus antitoxin in addition to the same amount of bacterial culture. The test guinea pig died during convulsions three days after the injection. The control guinea pig remained lively for 30 days. The test was identically repeated one month later with two pairs of guinea pigs, using the same culture. One of the guinea pigs died within eight days, whereas the other showed slight symptoms of disease beginning with the ninth day, but recovered in two weeks from the illness which appeared severe. No disease symptoms whatsoever appeared within 30 days in the control guinea pigs. The efforts to isolate a pure strain of the *Cl. tetani* were later succesful and mouse tests were carried out. The first test was made with three pairs of mice, which all received 0.1 cc. of a 48-hour old liver broth culture, in addition to which one-half of them were given 0.1 cc or 100 units of tetanus antitoxin. All the test mice died in less than 22 hours, whereas the control animals remained lively for 30 days. The test was repeated

with one pair of mice in the same manner except that 0.1 cc of liver broth culture dilution (one normal loopful of 24-hour old liver broth culture in 0.5 cc of physiological saline solution) was administered on each mouse. Within two days the test mouse fell ill, dragging its hind legs, its stomach bloated and back arched. The mouse died in 2 days and 21 hours with its back bent, receiving several cramp attacks before death. The control mouse remained lively for 30 days. A sample was excised post-mortem from the laparotomy wound of the above mentioned patient in the presence of the author, in addition to which samples were removed from the uterus stump in as sterile a manner as possible. Only *Staphylococcus aureus* was found in the bacteriological examination.

In the seventh case (No. 7/47) bacteria morphologically resembling tetanus bacilli and anaerobes of the *Cl. bifermentans* type grew from the investigated catgut. A mouse test with three pairs of mice was made with a mixed culture of these bacteria in liver broth. All test mice died within three days, while the control mice remained lively for 14 days. Isolation of tetanus bacilli from this mixed culture was not succesful and almost 100 metres of the catgut samples had to be investigated before *Cl. tetani* was discovered again. It was isolated and a mouse test with three pairs of mice was made with the pure culture by administering to the first pair 0.1 cc of a 24-hour old liver broth culture, to the second pair 0.1 cc of a dilution of this culture (one loopful of culture in 0.5 cc of physiological saline solution) and to the third pair likewise 0.1 cc, but of a weaker dilution (one normal loopful in 2.0 cc physiological saline solution). The second mouse in each pair received furthermore 0.2 cc or 120 units of tetanus antitoxin (Sächsisches Serumwerk, Dresden). The control mice remained lively for 30 days, whereas the test mice died the sooner the stronger the suspension had been, *i.e.*, within 19 hours, 2 days, and 2½ days. *Cl. tetani* was also discovered in the piece of tissue submitted and a pure culture was made. A mouse test with three pairs of mice was performed from the pure culture as described above. The control mice were lively for 30 days, whereas the test mice died the sooner the stronger the suspension had been, or after 19 hours, 2 days 21 hours, and 5½ days.

The following two cases, (Nos. 8/47 and 9/47) occurred simultaneously in the same hospital. Seven unopened original cartons

containing catgut of the same thickness as had been used at the operation were submitted for examination. Bacteria morphologically resembling *Cl. tetani* were found in the two thickest catgut samples. A pure strain was isolated from one of these and mouse tests were carried out, starting with one pair. The test mouse died after 1 day 18 hours, the control mouse remaining lively for 30 days. The test was repeated with two pairs of mice, to both of which were administered 0.1 cc of liver broth culture (one normal loopful of 48-hour old liver broth culture in 0.5 cc of physiological saline solution), in addition to which the control mice received 0.2 cc or 120 units of tetanus antitoxin. After three days the test mice moved stiffly, with the back arched. After four days the right side of the stomach of one of the mice was distended, the right foot was crooked and moved poorly, the back was arched and scoliotic to the right. The mouse had cramp attacks lasting a few seconds at short intervals, and falling on its right side it died a few hours later. The other mouse also died within less than five days after suffering several cramp attacks. The control mice remained lively for 30 days.

The strain of tetanus bacillus type found in the other catgut was not isolated.

DISCUSSION

It appears from the literature that although numerous cases of postoperative tetanus have been reported and catgut frequently has been suspected as being the source of infection and bacteriological investigations have even been made, the tetanus bacillus has been isolated or demonstrated in rare cases only.

The total length of catgut examined in the investigations here reported was about 600 lineal metres. It may be mentioned by way of comparison that 10 packages with 25 metres of catgut and 10 packages with 50 metres of catgut were taken at random from the store at the factory which prepared those which had caused the infections. No anaerobic bacteria whatsoever were found in eleven of these packages at bacteriological examination. Undetermined anaerobic bacteria were found in nine of the packages over lengths of minimum one metre and maximum four metres, but no bacteria that would morphologically have resembled tetanus bacilli were discovered.

In some of the cases (Nos. 1/45, 2/45, 4/47, 5/47, 6/47 and 7/47) it might be assumed that the tetanus bacilli had access to the packages after opening, but on the other hand it should be borne in mind that the catgut was handled in large central hospitals and also to a large part of the present series in university clinics, *i.e.*, in places where sterility requirements can be met and it is endeavoured to meet them. For this and for other reasons, the possibility of outside infection cannot be regarded as probable.

In some cases, furthermore, (Nos. 3/46, 8/47 and 9/47), the catgut in the factory's original unopened and undamaged cartons was examined. In these cases it was demonstrated that the source of the tetanus might very probably be the catgut, in which the strains of tetanus bacilli were pathogenic to animals.

Tetanus bacilli were found in eight of the nine cases investigated. (In one case, it is true, (No. 1/45), final identification by animal test could not be performed, but anaerobic bacteria fully resembling tetanus bacilli were found in the catgut as well as in the wound excised at autopsy of the patient who died from clinical tetanus). *Cl. tetani* could be isolated from catgut samples submitted in seven cases and in addition from a tissue sample removed at autopsy in case No. 7/47. In one case only no *Cl. tetani* was found, but it is noteworthy that also in this case *Cl. welchii*, which is known to be pathogenic, was found. It likewise deserves to be noted that in addition to the tetanus bacilli some type of gas gangrene bacteria was found from the catgut in every case (*Cl. welchii* in cases No. 1/45, 2/45, 3/46, 6/47; *Cl. novyi* in cases No. 3/46, 4/47, 5/47, 6/47, 7/47, 8/47, 9/47; and *Cl. histolyticum* in case No. 7/47). According to Zeissler (44) the presence of gas gangrene bacteria has the effect of intensifying the pathogenicity of tetanus bacilli. Gas gangrene bacteria were found in two of the three tissue samples examined, *i.e.*, *Cl. welchii* in case No. 1/45 and *Cl. novyi* in case No. 7/47. In addition to the gas gangrene bacteria, apathogenic anaerobes were found in several catgut samples, such as *Cl. multifermentans*, *Cl. bifermentans*, *Cl. sphenoides* and *Cl. tertium*.

According to Matas (24) contamination by feces is the cause of tetanus following operations in the pelvic region. Cases No. 2/45, 5/47, 6/47, 7/47, 8/47, 9/47, 10/47, 11/47 and 12/47 of these series belong to this category. The probability of catgut being the source of infection was, however, demonstrated in the investi-

gations conducted on the basis of the material submitted in cases No. 5/47, 6/47, 7/47, 8/47 and 9/47, for which reason the writer would consider the probability of catgut infection more important in the present series than Matas's hypothesis.

Some investigators (9, 11, 27, 37 and 43) regard the intestinal system as the source of infection when it has been opened in connection with an operation. According to the literature, the contents of intestines opened at operation have been investigated in a few cases. For example, in one of Wohlgemuth's two cases (43) tetanus bacilli were demonstrated from the intestines by animal test. Such a result was to be expected on the basis of the studies of Buzello and Rahmel (3) and TenBroeck and Bauer (39). Knoblauch (17) likewise found bacteria which morphologically resembled tetanus bacilli in mucous membrane stainings of a necrotic intestine, but culture was not succesful, due in the opinion of the author, to faulty technique. Possibility of infection from the intestines may have been present also in the above mentioned cases of the present series. Cases No. 1/45, 2/45, 4/47, 8/47 and 10/47 belong to this category. However, the possibility of catgut infection has been demonstrated in respect to cases No. 1/45, 4/47 and 8/47, for which reason infection from catgut rather than from the intestines is more probable also in these cases. In would seem as if the concepts in the literature according to which the source of infection in postoperative tetanus would be the skin, the intestinal system itself or its contents ejected upon the skin were due to the catgut investigations having been negative and no other source of infection having been found. When dealing with bacteriological investigations it should, however, be borne in mind that a negative result gives no absolute proof. Such a vast series of cases with negative findings as that described by Bulloch (2) tends to prove the innocence of catgut, but, on the other hand, the negative results are understandable for the reason that tetanus bacilli are extremely difficult to demonstrate even in comparison with other anaerobes. It should also be remembered that the technique of anaerobic bacterial cultivation has made great progress since the older reports. In any case the isolation of tetanus bacilli requires a very great amount of time, patience and work. The writer has spent at least four months merely in the isolation of each strain of tetanus bacilli.

In view of the above described series of cases and of other catgut investigations carried out by him the author fully agrees with Clock (6) in the opinion that chemically sterilised catgut should be abandoned and that as strict a sterility control of catgut as possible should be made.

The writer is of the opinion, on the basis of this series of cases and of other sterility investigations on catgut which will be described in greater detail later, that the role of catgut in the infection mechanism of postoperative tetanus is greater than is shown by the literature.

SUMMARY

1) The writer has described thirteen postoperative cases of tetanus, ten of which ended fatally and three recovered and in all of which chemically sterilised catgut was suspected to be the source of infection.

2) The catgut was examined in nine cases. In every case it was found to be unsterile in at least some of the samples submitted, which partly were opened and partly original cartons.

3) In eight cases tetanus bacilli were found in the catgut frequently from the thickest kind, (in one case, however, definite identification by animal test could not be performed). In seven cases it was possible to isolate a pure culture of tetanus bacilli from the catgut and furthermore in one case from a tissue sample, and to demonstrate their pathogenicity to animals.

4) Gas gangrene bacteria, such as *Cl. welchii*, *Cl. novyi* and *Cl. histolyticum*, known to intensify the pathogenicity of tetanus bacilli were found in the catgut samples submitted in each case.

5) On the basis of the series of cases presented as well as of other sterility investigations on catgut (to be described in greater detail in later reports), the writer is of the opinion that catgut plays a more prominent part in the infection mechanism of postoperative tetanus than is generally presumed.

6) Investigations to determine tetanus bacilli in catgut are very difficult and require much time, which may to some degree explain the predominance of negative results among cases described in the literature.

7) The writer fully agress with Clock (6) that chemically sterilised catgut should be abandoned and that as strict attention as possible should be devoted to the control of the sterility of catgut.

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FROM THE STATE SERUM INSTITUTE, HELSINKI

SYPHILIS ANTIGEN ISOLATED FROM WHEAT EMBRYOS

PRELIMINARY REPORT

By

EERO UROMA and M. TUOMIOJA

(Received for publication December 29, 1949)

Syphilis antigen has been extracted from various vegetable tissues: potatoes (1), peas, beans and corn (2), olives and coconuts (3). Stevenson (4) reported 1949 on an antigen extracted from soya bean. With this antigen he obtained results which were in good agreement with Wasserman and Kahn results. The extracts he used as antigen were unpurified alcoholic extracts and could not be stored more than thirty-six hours, after which they became too sensitive.

A substance, »Sitolipin», has been extracted and purified from wheat embryos. It gives a good antigenic response when used in very small concentrations. The antigen can be stored by room temperature.

Sitolipin is soluble in alcohol, colourless, unsaturated compound, containing nitrogen and phosphorus.

The antigenic activity was examined by a method similar to the VDRL tube test. By altering the relations between sitolipin, lesitin and cholesterin it is possible to standardize the antigen to a wanted level of sensitivity.

The results summarized in Table 1 have been obtained by an antigen in which the relations of sitolipin, lesitin and cholesterin were 3 : 40 : 90. The antigen can also be used in a slide test like the cardiolipin antigen.

TABLE 1

UR	VDRL	Kahn	No. of Sera
+	+	+	78
+	+	−	13
+	−	+	1
−	+	+	1
+	−	−	7
−	+	−	3
−	−	+	4
99	95	84	107
−	−	−	283
			390

Table 1 shows the results obtained with sitolipin antigen, »UR-test», with the VDRL tube test and with the Kahn test. From 390 tested sera 283 were negative in all the three tests and 107 positive at least in one of the tests. Of the positive sera, 99 were UR-positive, 95 VDRL-positive, and 84 Kahn-positive.

The work is being continued with a view to elucidating the chemical nature of sitolipin and its value in clinical use as antigen for syphilis.

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PAPERS READ AT THE THIRD SCANDINAVIAN CONGRESS OF PHARMACOLOGISTS

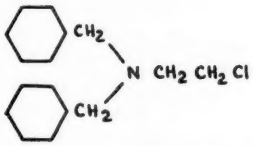
HELSINKI

SEPTEMBER 2—3, 1949

E. Askelöf and B. Sundbeck, Stockholm

NEW DIBENAMIN DERIVATES

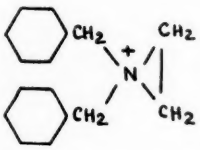
During the years 1945—49, Nickerson and Goodman published the results of a pharmacological investigation concerning a new type of adrenergic blocking agents whose mother substance was »dibenamin».



(1)

N-N'-Dibenzyl-β-chloroethylamine

Nickerson connects the antiadrenergic effect to the capacity of the dibenamin compounds to form the following intermediates:



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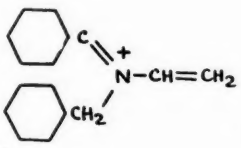
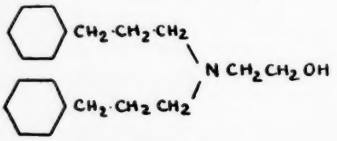


(3)

In this connection the hypothetic compound (3) would block the »receptor substance» of the sympathomimetic agents. A compound of this type is stabilized through the hyperconjugation stated in the formula.

In the course of investigations concerning antispasmodics, the writers are at present examining some compounds that possess a constitution suggestive of dibenamin and, owing to their chemical and pharmacological properties, may contribute towards an elucidation of the mechanism of dibenamin.

The first group of substances to be studied is the bisaralkylamines which may be represented by

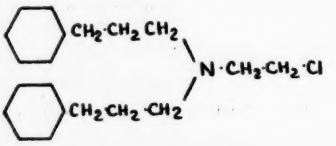


(4)

bis-γ-phenylpropyl-β-hydroxyethylamine

Even with varying numbers of C atoms in the alkyl chains, these compounds are antispasmodics of the same type and strength as papaverine. This applies also to many of the alcohols corresponding to the dibenamin derivates, e.g., dibenzylaminoethanol and naphtylmethylethylaminoethanol. The forementioned compounds are no more antiadrenergic than these alcohols.

Nor does the β-chloroethylamine, corresponding to compound (4).



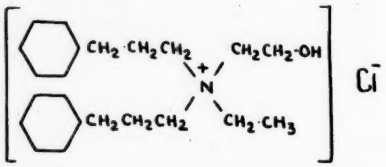
(5)

bis-γ-phenylpropyl-β-chloroethylamine

shows any antiadrenergic effect, though in this instance a quaternary compound and a reactive derivate, stabilized through hyperconjugation, corresponding to (2) and (3), respectively, are conceivable.

In connection with these investigations we have made attempts at alkylating (4) and (5) into quaternary compounds.

From (4) was obtained

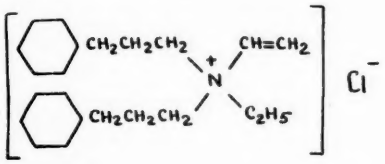


(6)

bis-γ-phenylpropyl-β-hydroxyethyl-ethylammon-
-iumchloride

which kept its antispasmodic effect, though without showing any antiadrenergic effect.

From (5), quaternization according to the same method produced a compound of the probable formula of



(7)

bis-γ-phenylpropylvinylethylammoniumchloride

This compound maintained its antispasmodic properties while, in addition, manifesting an antiadrenergic effect of the same magnitude and duration as dibenamin.

The investigations regarding the question how the variations in the length of the aralkyl chain affect the rate of quaternization of the bisaralkyl-β-chloroethylamines their self-quaternization and the stability of the quaternary compounds and, further, what influence these factors have upon the antiadrenergic effect, will be continued. [1]

[1] The tests of the antiadrenergic effect have been performed by Dr. E. Nyman, Assistant Professor, and E. Östlund, med. cand., of King Gustaf's Research Institute in Stockholm. The spasmolytic effect on guinea-pig intestine has been determined by A. Raud, med.cand., of the Physiologic Institution at Uppsala.

Arvid Carlsson and Folke Serin, Lund

VARYING TOXICITY OF NIKETHAMIDE

In an investigation of certain problems concerning the toxicity of nikethamide we tested two samples manufactured by different synthetic methods. Both preparations conformed to the standards for purity prescribed by the Swedish Pharmacopeia.

One of the preparations was, however, found to contain a surface-active impurity. Toxicity tests on tadpoles revealed that high concentrations of the less pure preparation were far more toxic than corresponding concentrations of the other preparation. Tests performed on mice, however, showed no distinct difference in the toxicity of the two substances.

The preparations showed no difference as regards hemolytic effect, local irritation, and the appearance of unanesthetized rabbits poisoned by intravenous injection, nor with regard to respiration, the arterial and the venous blood pressures of anesthetized (urethane) rabbits. Neither did Coramin (Ciba), which was used as a control, differ in any of the above respects.

These experiments show thus that nikethamide preparations satisfying the standards of the Swedish Pharmacopeia may contain impurities influencing their pharmacodynamic effect.

In the above tests on white mice the toxicity varied considerably from one occasion to another. The variations could not be attributed to any of those factors long known to be capable of affecting the results of toxicity tests.

A careful analysis of the material (930 mice) revealed that the mortality rate varied according to the time of the day the injections were given.

In animals injected with nikethamide some time between 12 a.m. and 6 p.m. the death rate was distinctly higher than in those injected some time between 6 p.m. and 12 p.m.

In view of this observation we made a systematic investigation

of the toxicity of nikethamide administered at various times of the day. In this investigation Coramin (Ciba) was used throughout. Each animal was given a uniform dose of 300 mg/kg nikethamide, subcutaneously, in the form of a 1 per cent aqueous solution.

Five groups of 25 animals each were injected at 2 a.m., 8 a.m. 2 p.m., 8 p.m., and 2 a.m., respectively. The mortality was found to vary according to the time of the day the injection was given. The rate of mortality was lowest, 22 per cent, after the injection given

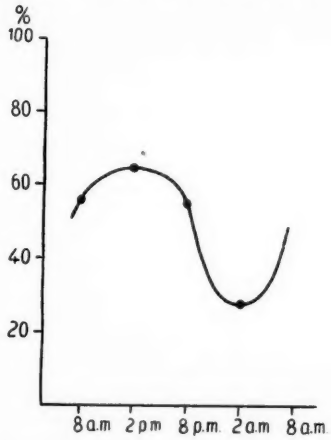


Fig. 1. — The toxicity of nikethamide in white mice at different times of the day. Absciss: time of the day. Ordinate: mortality per cent.

at 2 a.m., the death rate following the other injections averaging 59 per cent. The difference, 37 ± 8.2. per cent is highly significant.

In another experimental series performed under analogous conditions, but with 40 mice in each group, the death rate was likewise lowest in animals injected at 2 a.m. and highest in those injected at 2 p.m. The difference between the minimum mortality and maximum mortality was significant, 35 ± 10.5 per cent.

In the last two experimental series the average mortality rates per 24-hour-day were practically equal, 53 per cent and 50 per cent respectively. The death rate of the total material is depicted graphically in Fig. 1.

A distinct correlation was found between the spontaneous activity and the mortality curve. The spontaneous activity was least at 2 p.m. and greatest at 2 a.m., whilst the mortality was lowest at 2 a.m. and highest at 2 p.m.

Ottar Dybing, Oslo

COMPARATIVE INVESTIGATIONS ON CURARE AND MYANESIN IN COMBINATION WITH HYPNOTICS

In a combination of two hypnotics, the paralyzing effect of the substances on respiration usually increases to the same degree as does their anaesthetic effect. A combination of curare with anaesthetics should theoretically give better anaesthesia due to the different mode of action of the periferally-acting curare and the central depressant hypnotics. In toxicity tests on mice d-tubocurarine and chloral hydrate have acted quite independently; the lethal doses of d-tubocurarine in mice treated with 0.6 mg/g of chloral hydrate intraperitoneally (LD 50) are a little higher (LD 50 = 1.6 μg d-tubocurarine intravenously pr. g mice) than in untreated animals (LD 50 = 1.3 μg/g). In mice injected intraperitoneally with 0.1 mg/g of pentobarbitone sodium (LD 50), on the other hand, the lethal doses of d-tubocurarine are decreased (LD 50 = 0.75 μg/g).

TABLE 1

TOXICITY OF THE COMBINATION CHLORAL HYDRATE MYANESIN

Chloral Hydrate	Myanesin mg/g	Number of Dead Mice
0.48	0.1	1 out of 15
0.40	0.1	0 » » 4
0.40	0.2	6 » » 10
0.40	0.4	11 » » 11
0.36	0.2	8 » » 15
0.24	0.3	7 » » 10
0.20	0.2	0 » » 4
0.20	0.4	4 » » 4
0.12	0.4	4 » » 4

Myanesin in combination with chloral hydrate had an action similar to that known from the combination of two anaesthetics, with central action, the toxicity of the two substances showing a typical synergism.

In Table 1 and Fig. 1 the additive action of chloral hydrate and myanesin is demonstrated. The addition line is drawn between LD 50 for chloral hydrate 0.6 mg/g and the corresponding figure for myanesin 0.5 mg/g.

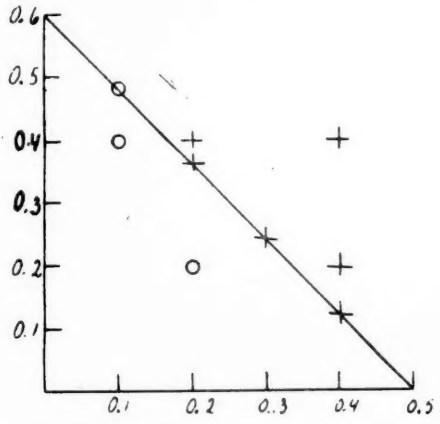


Fig. 1. — Toxicity of the combination chloral hydrate + myanesin.
+ Lethality above 50 per cent
O Lethality below 50 per cent

Daniel Eckert, Matti Paasonen,
and A. Vartiainen, Helsinki

ON HISTAMINE IN GNAT (*Culex pipiens*)

Reactions to bites of insects show features which suggest that they are to be ascribed to histamine or to biologically histamine-like substances. It has been proved that the poison of some insects contains histamine and that the poison of some other insects liberates histamine from animal tissues.

We noticed that a gnat's bite caused a Lewis triple reaction on the skin similar to that caused by histamine in a concentration of 1: 1000—1: 2000. Since it seemed possible that the substance or substances responsible for this reaction came to the wound from the gnat, we decided to study whether the different parts of the body of the gnat contained either histamine or other substances with histamine-like action.

It was found that extracts made by Code's method from gnats and their parts contained a substance which caused contraction in the intestine of the guinea-pig. Biological analysis showed that this substance was either histamine or some other substance which had a very close resemblance to histamine. Antistin, a histamine antagonist, was found to inhibit contraction caused by this extract in the same concentration as it inhibited a corresponding contraction caused by histamine. Histaminase (Torantil) inactivated the extract exactly in the same way as it inactivated histamine, while atropine did not prevent contraction caused by this extract.

A series of experiments revealed an average of 0.192 γ of histamine in the gnat. Table 1 records the amounts of histamine found in the different parts of the body of the gnat. It is particularly worthy of note that the head of the insect contains proportionately six to seven times more histamine than the other parts of the body.

TABLE 1

	γ	γ/mg
Whole Body	0.192	0.074
Head	0.049	0.410
Middle Parts	0.091	0.057
Hind Parts	0.052	0.070

Assuming that histamine is transferred from the gnat into the tissues stung by it, there should be a corresponding decrease in the histamine content of the insect. In order to see whether this was the case, we first allowed the gnat to pierce the skin with its sting and then examined it before it had had time to suck blood. It was found that the histamine content of the head had decreased to 30 per cent of the original amount, while the amount deposited in the other parts of the body remained very much the same (Table 2).

TABLE 2

	γ	γ/mg
Whole Body	0.148	0.055
Head	0.014	0.120
Middle Parts	0.066	0.042
Hind Parts	0.068	0.068

The amount of histamine lost by the sting averaged 0.05 γ. One drop of 1: 1000 histamine solution (= 0.05 ml), injected into the human skin, contains exactly the same amount, 0.05 γ, of histamine and is usually sufficient to produce a definite histamine response.

It seems highly probable that the gnat injects through its sting histamine, a powerful vasodilator, into the capillary, to facilitate sucking and to reduce the dangers connected with it.

DANIEL ECKERT and OSMO VARTIAINEN, Helsinki

DESENSITISATION WITH HISTAMINE

On the basis of experiments described in the literature (2–7), histamine in intravenous infusions was administered to patients suffering from asthma. To 500 ml of saline were added 1.7 ml of 1:1000 histamine dihydrochloride (Imido »Roche»), which corresponded to ca 1 mg of histamine and 2.5 ml of heparin. The infusion was carried out every second day during fortnight, and the speed of infusion was increased on each occasion according to the patient's tolerance. Only those patients were chosen as subjects who had no marked emphysema and whose sedimentation rate was normal. The present report is based on results obtained in five cases. In three of these the asthma was of allergic origin; in two the etiology remained obscure.

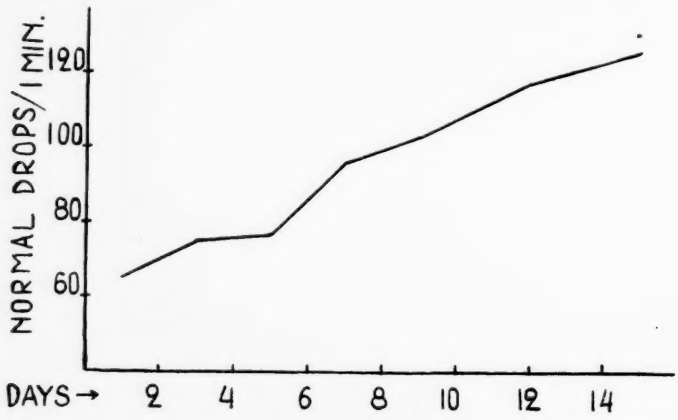


Fig. 1. — The increase in the maximal infusion speed (normal drops per minute) in intravenous histamine infusion as estimated by the subject's objective and subjective responses. The curve represents the average results obtained in a series of five cases.

The histamine tolerance of the patients was found to increase as the treatment progressed. This was evident from the fact that infusion could be accelerated. Fig. 1 illustrates the development of histamine tolerance in the light of average values obtained.

Such histamine symptoms as dyspnea, reddening of the face, erythema of the skin, and headache decreased with every successive infusion.

No significant change could be demonstrated in the white blood count. It seems, however, that the infusion of histamine reduces the number of eosinophil granulocytes. The percentage of eosinophilia in the subjects was greater before than after histamine therapy (Table 1).

TABLE 1

Case No.	Before Histamine Therapy		After Histamine Therapy	
	Leukocytes	Eosinophils %	Leukocytes	Eosinophils %
1	8.400	7.5	7.200	2,5
2	5,700	26,0	4.400	11,0
3	4.600	1,5	4.800	2,0
4	5.700	11,0	6.600	3,0
5	4.900	18,0	4.500	2,5

The rise in the blood sugar level reported in the literature could not be demonstrated with the Hagedorn-Jensen method used.

Histamine infusion caused slight alterations in the blood pressure. At the beginning of the first infusions a fall of 5—10 mm Hg was recorded, but otherwise no noteworthy alteration took place in the course of the infusion. Immediately after it the blood pressure rose, to return to normal within a few minutes. The fluctuations in the blood pressure decreased with each infusion.

In three cases the subject's histaminolytic power was determined by Ahlmark's method (1) before and after histamine therapy. Only in one case could a definite increase be demonstrated.

Patients with asthma were distinctly more susceptible to intravenously infused histamine than normal subjects. The symptoms of asthma decreased as the histamine tolerance increased. In two cases follow-up six month after the end of the therapy showed that the patient's condition was excellent, and in the other cases the symptoms and signs showed a distinct improvement.

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Erik Jacobsen, Copenhagen

THE CAUSE OF THE FORMATION OF ACETALDEHYDE AFTER INGESTION OF TETRAETHYLTHIURAMDISULPHIDE (ANTABUSE) AND ALCOHOL[1]

It has been demonstrated that the concentration of acetaldehyde in the blood after the intake of alcohol preceded by the administration of tetraethylthiuramdisulphide (Antabuse) is much higher than after the same amount of alcohol without the previous intake of Antabuse (Hald and Jacobsen 1948, Hald, Jacobsen and Larsen 1949). It has furthermore been shown that the symptoms observed after alcohol intake in persons having ingested Antabuse can be explained as due to this increase in the acetaldehyde concentration in the organism (Asmussen, Hald and Larsen 1948).

The investigations described in this paper give an explanation for the increased formation of acetaldehyde under these circumstances. The experiments were carried out on rabbits, partly on intact animals, partly with isolated surviving organs perfused with blood. First, it could be shown that the acetaldehyde formation takes place in the liver (Jacobsen and Larsen 1949). Rabbit livers were isolated and perfused with circulating defibrinated oxygenated blood by the method described by Niels Aage Nielsen (1933). Some rabbits were untreated, others were treated with 0.75 g Antabuse twice 28 and 20 hours prior to the experiment. When the perfusion was well established, alcohol was added to the perfusion blood and the concentration of alcohol and acetaldehyde in the blood determined with intervals. The results of typical parallel experiments are given in Tables 1 a and b.

[1] From the Biological Laboratories of Medicinalco Ltd., Copenhagen S.

TABLE 1 a

CONCENTRATION OF ALCOHOL AND ACETALDEHYDE IN BLOOD PERFUSED THROUGH THE ISOLATED LIVER FROM A NORMAL RABBIT

Time	mg % Alcohol in the Blood Leaving the Liver	mg % Acetaldehyde in the Blood Leaving the Liver
1.28 p.m.	(22)	(0.12)
1.42	(36?)	(0.44)
1.43 Addition of 0.48 g absolute alcohol in 5 ml Tyrode's solution to the perfusion blood.		
1.53	121	(0.17)
2.13	100	(0.18)
2.58	79	(0.11)
3.33	41	(0.16)
3.58	33	(0.13)

TABLE 1 b

CONCENTRATION OF ALCOHOL AND ACETALDEHYDE IN BLOOD PERFUSED THROUGH A LIVER FROM A RABBIT TREATED WITH ANTABUSE

Time	mg % Alcohol in the Blood Leaving the Liver	mg % Acetaldehyde in the Blood Leaving the Liver
1.29 p.m.	5	(0.12)
1.39	35	(0.13)
1.40 Addition of 0.52 mg abs. alcohol.		
1.55	127	3.14
2.10	126	3.40
2.40	103	4.48
3.41	59	3.83

It will be seen from this table that after addition of alcohol a considerable amount of acetaldehyde is formed in the livers of antabuse-treated animals. From the amount of blood perfused, the weight of the liver and the concentration of alcohol in the blood, it is possible to calculate the amount of alcohol combusted per minute. This amounts to an average of *2.3 mg per minute per 70 g liver* (for details see Jacobsen and Larsen). This figure will later be used in some quantitative considerations. No acetaldehyde formation after the addition of alcohol was found in perfusion experiments with isolated hind limbs of antabuse-treated animals. Hence it must be concluded that the muscles do not play any rôle in the acetaldehyde formation. The liver cells, not the blood,

must be responsible for the acetaldehyde formation, since livers from antabuse-treated animals form acetaldehyde when perfused with blood from untreated rabbits, while livers from untreated rabbits do not form acetaldehyde when perfused with blood from antabuse-treated rabbits.

Investigation on acetaldehyde metabolism is not quite simple. It is not enough merely to inject a single dose of acetaldehyde into an animal and measure the decrease of acetaldehyde in the blood. Under all circumstances acetaldehyde is so rapidly metabolized that is has disappeared from the organism long before any equilibrium between the blood and the body liquid is established. The only way to study the rate of acetaldehyde metabolism is to infuse acetaldehyde intravenously at a constant rate through a considerable time and study the proportion between the infusion rate and the concentration of acetaldehyde in the blood. This is done by Hald and Larsen (1949). A series of untreated and antabuse-treated rabbits were infused intravenously with acetaldehyde at a constant rate through 1 hour and more. At the end of this time acetaldehyde was determined in the blood. In spite of great individual variation, a clear difference could be shown between the results in the two groups. The average figures from these experiments are given in Table 2.

TABLE 2

AVERAGE RELATION BETWEEN ACETALDEHYDE DOSAGE AND CONCENTRATION IN BLOOD IN RABBITS TREATED AND NOT TREATED WITH ANTABUSE

mg Acetaldehyde Infused in Blood per Minute	mg % acetaldehyde in blood	
	in Untreated Rabbits	in Rabbits Treated with Antabuse
1	< 0.1	0.4
2	0.2	0.9
5	1.7	2.9
8	3.5	5.4

It will be seen that the rabbit organism is able to metabolize a rather high amount of acetaldehyde. In some experiments we have infused as much as 9 mg acetaldehyde per minute through 2 hours without any rise in blood acetaldehyde beyond the level reached after some 20 minutes. It was furthermore seen that the

animals treated with Antabuse are able to metabolize the same amount of acetaldehyde per minute as the untreated animals, but that the infusion of this amount results in a higher concentration of acetaldehyde in the blood. The ratio between the treated and untreated animals, however, decreases with increasing concentrations of acetaldehyde. In these experiments it decreased from more than 1: 4—5 when 1 mg per minute was infused to less than 1: 2 when 7 mg were infused. Although it can be concluded from these experiments that the acetaldehyde metabolism in the organism is delayed under the influence of Antabuse, it is impossible to decide whether this delayed metabolism is the only cause of the acetaldehyde formation after Antabuse + Alcohol. As a matter of fact the differences in the acetaldehyde concentration of the blood between the treated and untreated animals are smaller than those seen after alcohol ingestion. In order to decide whether this decreased acetaldehyde metabolism under the influence of Antabuse is the whole explanation of the Antabuse effect or only a part of it, it was necessary to make experiments with liver perfusion. To the circulating blood in the liver perfusion experiments was added acetaldehyde at a constant rate. When the circulation has proceeded so long that the equilibrium in acetaldehyde between the blood liquid and the cells is established it is possible from the concentration of acetaldehyde in the blood entering the organ, the concentration of acetaldehyde in the blood leaving the organ and the blood flow in ml per minute to calculate the amount of acetaldehyde metabolized per minute in the liver. This amount is

TABLE 3

AVERAGE RELATION BETWEEN THE METABOLIC RATE OF ACETALDEHYDE AND THE CONCENTRATION OF ACETALDEHYDE IN BLOOD PERFUSED THROUGH ISOLATED LIVERS OF UNTREATED RABBITS AND RABBITS TREATED WITH ANTABUSE

mg Acetaldehyde Metabolized per Minute	Average Increase of mg Acetaldehyde per 100 ml Perfused Blood in:		Ratio $\frac{a}{b}$
	Untreated Rabbits a	Rabbits Treated with Antabuse b	
1	0.02	0.75	37
2	0.08	2.35	29
3	0.40	4.35	11
4	0.90	6.75	13
5	1.60	9.20	6

adjusted to the average liver weight of 70 g and compared with the concentration of acetaldehyde in the blood leaving the liver, which must be in equilibrium with the concentration in the liver cells. A series of experiments made after this principle was made with livers from untreated rabbits and rabbits given Antabuse in the described way [Hald, Jacobsen and Larsen (1949 II)]. The average results from these experiments are seen in Table 3.

As mentioned, it is possible to calculate the rate of alcohol metabolism in livers perfused with blood + alcohol. Under the assumption that all alcohol is oxidized according to the formula:

$$\begin{array}{l} CH_3 \\ | \\ CH_2OH \end{array} \quad \xrightarrow{-2H} \quad \begin{array}{l} CH_3 \\ | \\ CHO \end{array}$$

it is possible to calculate the amount of acetaldehyde produced per minute in these livers and compare this figure with the concentration of acetaldehyde in the blood perfused through the liver. This is done in Table 4.

A comparison between the figures given in Tables 3 and 4 shows that the concentration of acetaldehyde in the livers perfused with alcohol is about the same as could be expected if the liver

TABLE 4

FORMATION OF ACETALDEHYDE IN BLOOD TO WHICH ALCOHOL HAS BEEN ADDED PERFUSED THROUGH NORMAL RABBITS' LIVERS AND ANTABUSE TREATED RABBITS' LIVERS. THE VALUES IN COLUMN. 2 ARE CALCULATED ON THE ASSUMPTION THAT ACETALDEHYDE IS AN INTERMEDIARY METABOLITE IN THE ALCOHOL CUMBUSTION

Liver from Rabbits	mg Alcohol Combusted per Minute per 70 g Liver	Calculated mg Acetaldehyde Equivalent to the Quantity of Alcohol Combusted per Min. per 70 g Liver	mg % Acetaldehyde in Perfused Blood	
			after 30 min.	after 120 min.
normal	2.03	1.94	0.20	0.16
	1.80	1.70	0.12	0.33
	3.19	3.05	0.22	0.25
treated	2.50	2.37	4.50	3.30
with	2.12	2.03	4.36	3.80
Antabuse	2.12	2.03	4.57	4.20 [1]

[1] After 60 minutes.

were perfused with the same amount of acetaldehyde per minute. The figures here given are averages falling within the observed biological variation.

From these results it must be concluded that the acetaldehyde formation after alcohol in Antabuse-treated animals is due exclusively to the delayed acetaldehyde metabolism under the influence of Antabuse.

It has now been shown by Kjeldgaard (1949) that Antabuse *in vitro* has an inhibitary effect on liveraldehyde oxidases, in concentrations of 0.1 μg per ml. This is not only observed for acetaldehyde, but also for a series of other aldehydes. This observation seems in combination with the experiments here given to present the full explanation of the Antabuse effect.

Even in another point Kjeldgaard's and our results are in conformity. From the experiments summarized in tables 2 and 3 it is seen that both the untreated animals and those treated with Antabuse are able to metabolize the same amounts of acetaldehyde, but the concentrations in the tissues required to metabolize the same amount of acetaldehyde are higher in the treated animals than in the untreated ones. It is also seen that the ratio between the increase in acetaldehyde concentration of the blood of untreated and treated animals are decreasing when higher amounts of acetaldehyde is metabolized. Kjeldgaard's in vitro experiments show that the relative inhibition of aldehyde oxidation by Antabuse decreases with increasing concentrations of aldehyde. He concludes that some competitive action must be present between acetaldehyde and Antabuse in the system.

SUMMARY

The acetaldehyde formation seen after alcohol intake in organisms treated with Antabuse (tetraethylthiuramdisulphide) takes place in the liver.

The increased concentration of acetaldehyde under these circumstances is fully explained by an inhibitory effect of Antabuse on the acetaldehyde metabolism.

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E. Jokivartio, Helsinki

A STUDY OF THE EFFECT OF TETRAETHYLTHIRAMDISULPHIDE (ANTABUSE) ON THE METABOLISM OF LACTIC ACID IN THE HUMAN ORGANISM

(To be published in extenso in Acta Pharmacol.)

In 5 of the 15 alcoholic addicts examined the curve of the lactic acid values after Antabuse administration was similar to that before the use of Antabuse. In 10 of the examined cases the initial value of the lactic acid after rest was generally higher than in normal cases and cases of alcoholism before using Antabuse. Their lactic acid values did not rise in exertion tests and 8 of them showed a continuous decrease in the lactic acid values in all four blood tests. The conformity of the results excludes the possibility of experimental error or chance, and Antabuse must therefore have direct influence on the lactic acid metabolism.

Nothing definite can be said of the details of the mechanism, but the effect may be due to the inhibitor influence of Antabuse, as an enzyme poison, on the enzyme systeme which govern the lactic acid metabolism (P. E. Simola, 1949) especially during heavy work. Antabuse is known to have a preventive effect on the enzymic change of alcohol, so that the normal route is partly or completely blocked and alcohol remains on the acetaldehyde stage (Hald and Jacobsen, 1948).

In this connection I wish to present the following interesting observation. Alcoholic addicts under Antabuse treatment in Helsinki have found out that they can prevent the clinical symptoms caused by Antabuse+alcohol by taking first an aromatic iron solution containing among other things, 0.2 per cent iron, 12 per cent alcohol and 16 per cent sugar syrup. It has been known that thiuramdisulphide forms compounds with metals (I. R. Geigy A.-G. 1942 and 1948, and Hald, Jacobsen and Larsen 1948), and

TABLE 1

LACTIC ACID VALUES FOR ALCOHOLICS BEFORE AND AFTER TAKING ANTABUSE

Subject No.	Blood Specimens mmg%							
	Without Antabuse				With Antabuse			
	I	II	III	IV	I	II	III	IV
1	9	15	8	7	8	21	8	8
2*	6	10	9	10	7	18	14	7
3	8	12	6	5	9	14	14	8
4	4	11	—	6	5	10	7	8
5	10	22	20	13	11	32	10	—
6*	12	26	17	14	13	12	6	1
7	7	15	7	7	20	11	9	7
8	9	16	6	—	38	36	24	13
9	9	32	9	9	13	13	9	9
10	19	33	8	23	17	10	—	10
11*	9	16	18	5	34	33	27	14
12*	13	45	13	7	55	27	18	11
13*	20	33	29	5	8	8	—	4
14*	18	36	—	14	23	15	22	22
15*	14	24	15	9	16	2	14	—

* Standard error $\pm$ 7 mmg% (due to impure reagents).

these compouns have been used in the destruction of pests. At the same time I wish to mention that one of the patients under Antabuse treatment at the Psychiatric Observation Hospital Ward in Helsinki, a metalworker, after having taken Antabuse and alcohol showed no usual clinic symptoms. Of the 5 alcoholic addicts of the Curve A type were all engaged in metalindustry: 2 filers, 1 painter and 2 masons. This suggests that an aromatic iron solution prevents the clinical symptoms because Antabuse forms complexes with iron and perhaps even with other metals. Hydrocyanic acid poisoning is similarly counteracted by means of copper (K. AGNER, 1939 and C.-J. CLEMENSON and B. NORBERG 1945).

Investigations on these reactions are at present in progress in the Department of Legal Medicine, University of Helsinki (HJELT and TAMMINEN), as it has been stated that Antabuse forms complexes with metals (iron, copper, nickel, cobalt and mercury) in ether-, chloroform- and alcohol solutions.

Harry Lybeck, Helsinki

EFFECT OF ATROPINE ON DENERVATED TISSUES

The writer, who has carried out experiments on white mice, describes the reaction of atropine sulphate on blocked extremities. A section was made on one sciatic nerve, while the other was left intact. Beginning with the day following the operation, the mice were given subcutaneous injections of atropine sulphate (up to 10 mg per day). The peripheral parts of the blocked extremities soon began to show degeneration. Continued medication intensified the process. Distinct histological changes were found, especially in the bone, bone-marrow and capillaries.

When the administration of the drug was discontinued, regeneration set in, but most of the blocked extremities remained in a state of invalidity.

GUSTAF ÖSTLING, Helsinki

A SIMPLE METHOD OF OBTAINING SERUM FROM BLOOD WITHOUT CENTRIFUGATION

Blood from a vein is directly put into a glass tube (*e.g.* 20 mm in diameter), one end of which is closed by a stopper. The other end is then closed by another stopper to which a wooden stick or a glass rod of the same length as the height of the blood column has been fastened. The tube is then immediately inverted and the blood allowed to coagulate.

The tube is again inverted; the clot remains hanging between the rod and the glass wall. The serum is pressed out by retraction. While it runs down the tube a certain number of red cells usually follow, forming a thin red layer on top of the stopper. The clear serum is tapped by allowing it to flow slowly out between the cork and the glass tube.

M. Andreassen (Dissert., Copenhagen 1943) has determined the retraction time of blood clot with the help of a method based in principle on the above.

GUSTAF ÖSTLING, Helsinki

THE EFFECT OF LARGE DOSES OF DIGITALIS ON AN EMPTY STOMACH

During the eighteenth and nineteenth centuries it was a common belief that digitalis vomiting was caused by local irritation of the stomach. In the beginning of this century Hatcher and co-workers showed convincingly by tests with animals that this was not the case. Yet many practitioners continue to prescribe digitalis to be taken after meals.

In order to ascertain whether *Folium digitalis purp.* really irritated the stomach or not, fifty patients with congestive heart failure were administered one gram of digitalis (ten tablets of 0.1 gram each) on an empty stomach at six o'clock in the morning. Only two patients had vomiting. This implies that local irritation of the stomach is rare in cases of oral administration of digitalis and that a maintenance dose may be prescribed once a day irrespective of meals.

Frederik Paulsen, Stockholm

ON THE CORTICOTROPIC HORMONE (ACTH)

The present situation in ACTH research i discussed. In Stockholm it has been possible to prepare ACTH from the pituitary residue after extraction of thyrotrophin and gonadotrophin. The yields were about the same as from fresh pituitaries. The main difficulty in the purification of ACTH is the high vasopressin content of the pituitary extracts. A method has been found to get rid of the vasopressin. Highly active preparations, suitable for clinical use in doses of 25—40 mg daily have been prepared and given dramatic results in therapeutical trials, not only in chronic arthritis but also in many other diseases. The mechanism by which ACTH exerts its effects is obviously not specific against the cause of any disease investigated until now. The effect is very general and of short duration. All metabolic processes appear to be altered to make possible a maximal reaction of resistance.

P. Peltola, Helsinki

THE EFFECT OF THYROID POWDER ON ADRENALIN TOLERANCE

The most frequently used methods for standardisation of the thyroid preparations at present are as follows:

— acetonitrile reaction, based on the heightened acetonitrile resistance of mice after thyroid feeding,

— axolotl reaction, based on increased metamorphosis of axolotls in water containing thyroid powder, as well as the reactions based on

— loss of weight and

— increased oxygen consumption, produced by thyroid powder.

All of these methods, however, have their own characteristic drawbacks and none of these has proved sufficiently accurate and reliable, which is best illustrated by the great number of methods in existence.

In my experiments pertaining to this problem I have attempted to find a method based on reaction similar to that elicited by thyroid preparations in man, and which would be independent, within the limits of possibility, of the technical performance and interfering factors. In the course of my studies, I have e. g. come upon the effect of sensitization to adrenalin exercised by thyroid preparations. This has been known of old, and attempts have been made to use it for standardization, e.g. through the effect to blood pressure (Kraus, Asher, Oswald) and by means of the Trendelenburg frog (Asher, Eiger, Csilla), but the methods are abandoned owing to the non-specifity of the effect. On the other hand, there are no reports on experiments based on variations of the lethal adrenalin dose subsequent to the effect of thyroid powder, and therefore I have deemed it advisable to carry out some experiments with this method.

These tests are performed on white fully developed male mice. 1124 test animals have been used so far. The experiment itself is

performed in the following way: while the test lasts, the mice are kept on a diet containing only wheat bread and water. They are allowed to ingest both ad libitum. The food left uneaten is removed in the morning and a few hours later the thyroid powder is administered to the animals in a water suspension, injected into a small quantity of wheat bread readily consumed by the mice; additional food is not given before the evening and after having checked that the selices containing thyroid suspension had been eaten up entirely. The adrenalin injections are applied subcutaneously, in a concentration of 1: 1000.

The results of the experiments brought to light e.g. the following facts:

the lethal dose of adrenalin varies in mice from about 5—9 mg/kg, 50 per cent mortality (LD50) about 6—7 mg/kg. It is relatively independent from accidental factors. Up to the present I have been able to show that the results are considerably affected by light, and to some extent by temperature in which the animals are living; but no influence is exercised by diet, season or sex. On the other hand, some variations can appear in different strains of mice, yet the 50 per cent mortality dose does not rise above 8 mg/kg (LD50). If the same strain is used, such considerable variations do not appear.

The thyroid powder, if administered to the mice for several days, induces an considerably lovered tolerance to adrenaline, as shown by the following Fig. 1.

The thyroid powder suspension is given to the animals for 12 days before applying the adrenalin injection. In addition, it seems to be clear that up to a certain limit the effect depends on the dose (up to 9 mg/20 gr), but very massive doses do not anymore alter the tolerance, a phenomenon which has been observed e.g. when following up the variations in the oxygen consumption of mice.

I have also performed experiments in order to study to what agent the change in the adrenalin tolerance brought about by thyroid powder is due. The results are as follows: the influence reducing the adrenalin tolerance begins about 4—8 days after the administration of thyroid powder and remains constant as long as the identical dose is used, irrespective of continual feeding of the powder, which is fully consistent with the observations made on variations of the oxygen consumption, Fig. 2.

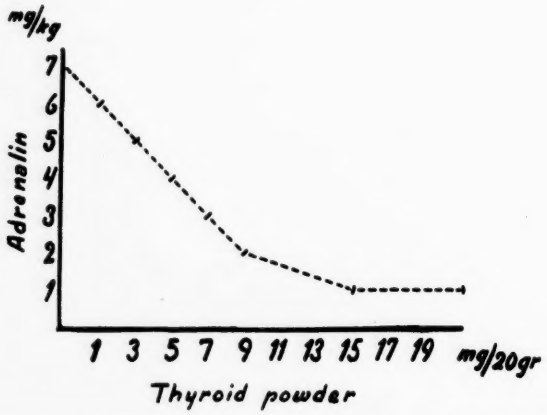

Fig. 1. — The total number of animals was 224. Determination of each point was made on 28—36 animals

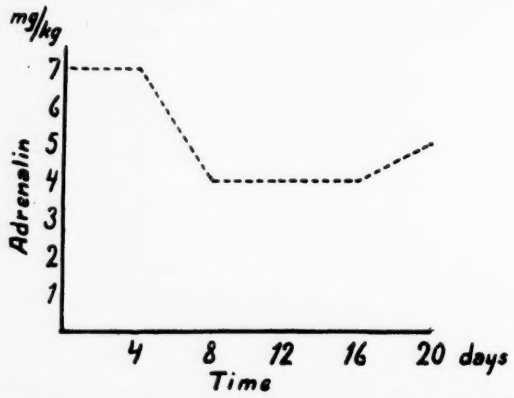

Fig. 2. — 5 mg of thyroid powder per 20 gr of body weight for 0—20 days

On the other hand, the lowered adrenaline tolerance induced by thyroid powder, appears to be unaffected by light, diet season and sex, depending instead to some extent on temperature.

Since the divergencies in the lethal dose of adrenaline and in the effect of thyroid powder on it are very little, and relatively insignificant variations in the dose of thyroid powder, to the extent of about 30 per cent, induce a pronounced change in the tolerance, it is evident, that a method for the standardisation of thyroid preparations can be developed on the basis of this reaction, particularly as it is possible to intensify its sentivity. Studies on this problem are still in progress.

Carl G. Schmiterlöw, Stockholm

SOME REMARKS ON THE DETERMINATION OF HISTAMINE, WITH SPECIAL REFERENCE TO HORSE BLOOD

It has been previously suggested by Åkerblom (1941) that the method of extracting histamine from blood and other tissues originally described by Barsoum and Gaddum and later modified by Code may involve an error, since histidine, if present in the extracted tissues, may be decarboxylated to histamine.

In order to avoid this error, a method has been devised in which the extraction is made with acidified alcohol. A comparison between the two methods has been made.

When pure histidine solutions were extracted according to Code's method, histamine appeared in the final extract. When the concentration of histidine in the solution was 1 mg per ml, the yield of histamine was on an average 0.50 ± 0.14 µg per ml. The histamine-identity was proved in the intestinal bath by means of antihistamines in specific doses. When extracting histidine solutions according to the alcohol method no histamine appeared in the final extract.

When extracting whole horse blood according to Code, the content of histamine was found to be 0.13 ± 0.01 µg per ml. When using the alcohol method, the yield was only 0.05 ± 0.01 µg per ml. This difference could be explained as being due to decarboxylation of histidine present in the hemoglobin. When extracting pure hemoglobin solutions according to Code, histamine appeared in the final extract in an amount which could explain the higher value obtained when extracting whole blood by the method of Code.

The alcol.ol extraction method does not involve the danger of decarboxylating histidine. The method is simple and also has the advantage that it is time-saving, the whole preparation being finished in about 1 ½ hour.

A. VARTIAINEN, Helsinki

SYMPATICOLYTICS

A review.